Centro de Investigación en Alimentación y Desarrollo, A. C.

CARACTERIZACIÓN DE LAS FORMAS NATIVA Y MUTANTES DE LA GLUTATIÓN S-TRANSFERASA CLASE Mu DE

CAMARÓN BLANCO Litopenaeus vannamei

POR

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Dedicatoria

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Resumen

Las glutatión S--transferasas (GST's) son una familia de enzimas detoxificantes que catalizan la conjugación del glutatión con una gran variedad de compuestos electrofílicos. Existen varios tipos de GSTs, que se diferencian por su especificidad hacia el substrato electrofílico y aunque todas comparten características estructurales generales, las diferencias en las secuencias primarias y por ende, en las estructuras de las enzimas, afectan sus propiedades catalíticas, Los aminoácidos Y6 y Y115 (en la mayoría de las GST clase mu) se han estudiado como los principalmente involucrados en las interacciones entre la enzima y el sustrato durante la catálisis de las GSTs, debido a la participación de su grupo hidroxilo en la activación y estabilización del glutatión (GSH), así como brindando asistencia electrofílica en la catálisis.

Para estudiar la importancia de estos residuos fue el sitio activo de la GST clase mu del camarón Litopenaeus vannamei, los residuos de tirosina Y7 y Y116 fueron reemplazados por fenilalanina por medio de mutagénesis sitio-dirigida. Las proteínas recombinantes de ambas mutantes (Y7F y Y116) así como la enzima silvestre se obtuvieron por sobreexpresión heteróloga en Escherichia coli y se purificaron por cromatografía de afinidad hacia GSH. De la misma manera se purificó una GST nativa clase mu a partir de branquias de camarón L. vannamei.

Se determinaron las constantes cinéticas de las enzimas utilizando CDNB como segundo substrato y se obtuvieron modelos estructurales de la enzima silvestre y las mutantes. Los valores de velocidad de catálisis (kcat) para la enzima silvestre recombinante resultaron muy similares a las reportadas para la GST clase mu purificada a partir de branquias. Sin embargo la eficiencia catalítica (Kcat/KmGSH) resultó mayor para la enzima recombinante, esto debido a la mayor afinidad (Km) que presentó esta enzima por el sustrato GSH.

En las mutantes la eliminación del grupo hidroxilo resultó en un ligero aumento en la Kcat de la Y116F y tuvo muy poco efecto en kcat-Km para GSH y CDNB, esto debido a la menor afinidad de la Y116.

rec()mbinanles, no se ()bservaron cambios importantes en la topologla del sitio activo al comparar las mutantes con la enzima silvestre.

En 9eneral, el cambio en los residuos Y7 y Y116 no so reflejó en los valores para las constantes cinéticas, lo cual podrla Indicar que la función que desemperian estos rnslduos en la catálisis, no es tan relevante para la GST de camarón. Esto podrla corroborarsi, por mr:idio do mutaciones en otros residuCls del sitio activo como la H108, pres, nte en el sitio activo de la GST clase mu de camarón a difornncia de otros organismos.

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Integración general

ANTECEDENTES

Catálisis enzimática y especificidad

Las enzimas son extraordinarios catalizadores, ya que pueden aumentar la velocidad de las reacciones químicas en un orden de 10^5 a 10^{14} sobre la reacción sin catalizar (Fersht, 1999). El poder de la catálisis enzimática resulta de la contribución de varios factores, tales como el de una unión y orientación precisa de los substratos en el sitio activo, la estabilización de las cargas y la disminución de la energía de activación en la reacción por medio de una mejor unión de un complejo intermediario (estado de transición) que la unión de los substratos, como lo propuso por vez primera Linus Pauling (Pauling, 1946).

Un ciclo catalítico (Figura 1) inicia con la formación reversible del complejo enzima sustrato (ES), seguido de una conversión química con una constante de velocidad k_2, que es seguida por la liberación del producto con la constante k_3.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P$$

Figura 1. Esquema general de una reacción catalizada por una enzima. Enzima (E) substrato (S) y producto (P). (Fuente: Silverman, 2000)

La estabilidad del complejo ES está relacionado con la afinidad del substrato por la enzima, la cual es medible por su K_s, que es la constante de disociación del complejo ES. Cuando k_2 es $<<$ k_{-1}, el término k_2 es llamado k_{cat} (la constante de velocidad catalítica) y a la constante de disociación K_s (k_{-1}/ k_1) se le denomina K_m (constante de Michaelis-Menten).

La constante de catálisis (k_{cat}), también llamada número de recambio, es el máximo número de moléculas de substrato convertidas a producto por sitio activo por unidad de tiempo. La constante de Michaelis-Menten (K_m) es una constante de disociación aparente de todas las especies de enzima unida (ES y EP en la figura 1). La constante de especificidad (k_{cat}/K_m) describe le eficiencia catalítica a bajas concentraciones de sustrato. Esta se puede usar para comparar la preferencia de la enzima por los sustratos que compiten. Este trabajo está basado en la determinación de la actividad enzimática que comúnmente se mide por medio de una cinética de estado estacionario (*steady-state*). Este tipo de análisis cinético provee información sobre los parámetros k_{cat}, K_m y k_{cat}/K_m.

Las enzimas no solo aumentan la velocidad de catálisis de una reacción, sino que también son específicas en la reacción. Algunas enzimas son altamente específicas para un substrato, tales como las aminoacil-tRNA sintetasas, responsables de la selección de aminoácidos durante la síntesis de proteínas (Fersht, 1999). Otras enzimas, en particular las involucradas en la detoxificación como las del complejo del citocromo P450 y las glutatión S-transferasas (GSTs), han evolucionado adquiriendo un amplio rango de especificidades para unirse a varios substratos, de manera que pueden actuar sobre una gran variedad de compuestos potencialmente tóxicos (Josephy y Mannervik, 2006). Algunos ejemplos de reacciones catalizadas por las GSTs se muestran en la Figura 2.

Enfrentando los retos celulares: Eliminación de compuestos tóxicos

De manera normal, los organismos están expuestos a compuestos químicos exógenos sin valor nutricio que son ingeridos, inhalados o absorbidos por el organismo y que pueden ocasionar daños. Estos compuestos, llamados xenobióticos, pueden ser dañinos para el organismo causando efectos tóxicos y algunas veces hasta carcinogénicos. Mientras que el reto impuesto a las células por estos compuestos ha aumentado en los últimos dos siglos debido a la exposición de químicos sintéticos introducidos por el hombre al ambiente durante ese tiempo, este reto probablemente

ha existido a lo largo de la propia evolución. Sin embargo, también en la naturaleza existen compuestos tóxicos, tales como toxinas fúngicas y de plantas, además de otros

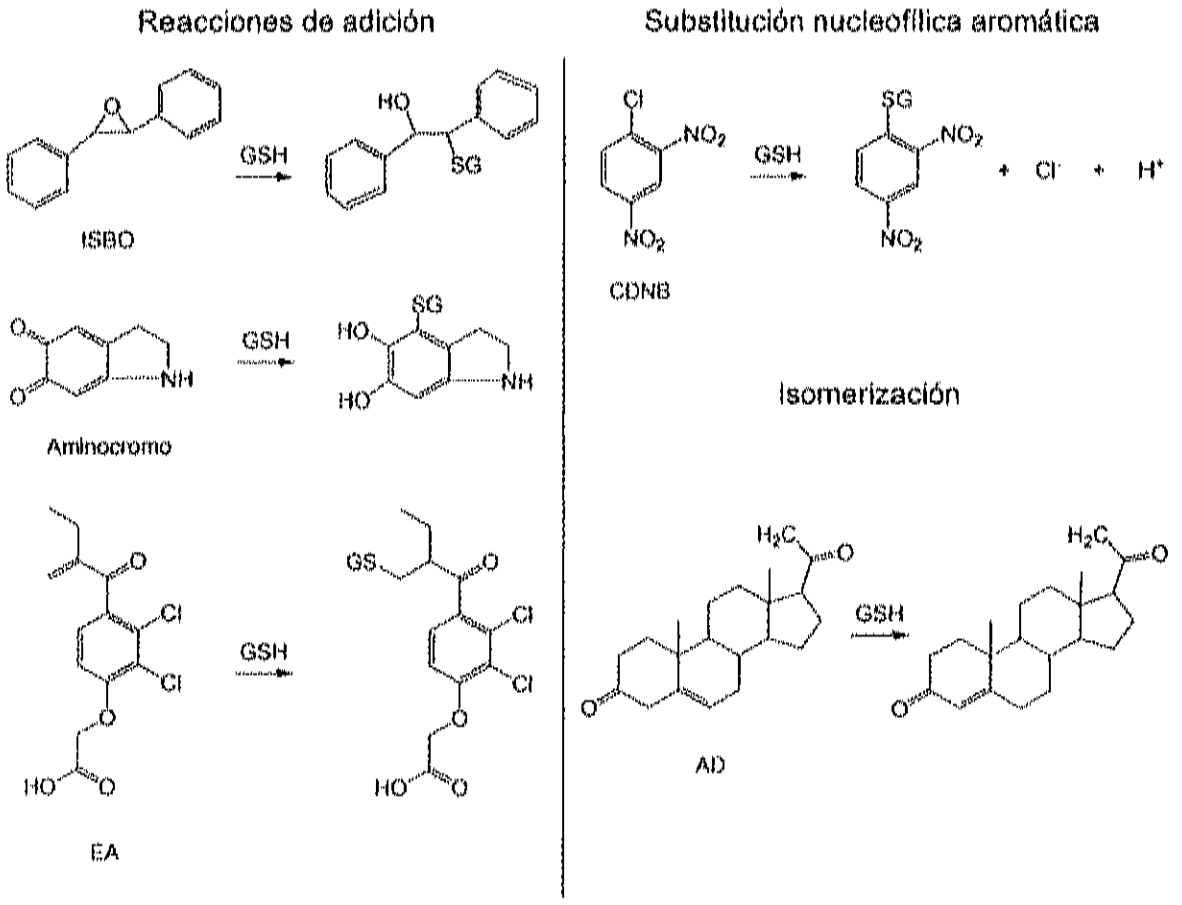


Figura 2. Las GSTs catalizan la adición del glutatión (GSH) a varios electrófilos, como en la abertura del anillo en los epóxidos (ejem. óxido *trans*-stilbeno, tSBO) y adiciones de Michael con aminocromo y ácido etacrínico (EA). La substitución aromática nucleofílica del 1-cloro-2,4-dinitrobenceno (CDNB) que ha sido extensamente estudiada. Las GSTs también actúan en la isomerización del 5-androsteno-3,17-diona (AD). (Fuente: Ivarsson y Mannervik, En prensa)

compuestos como las especies reactivas del oxígeno, donde se incluyen los radicales superóxido y peróxido de hidrógeno.

La habilidad para sobrevivir a este reto, causado tanto por los compuestos tóxicos producidos de forma endógena así como por los xenobióticos, probablemente representa una adaptación fundamental para la sobrevivencia. La biotransformación catalítica se ha desarrollado como un importante mecanismo bioquímico de protección contra especies químicas tóxicas. Las células poseen un impresionante arreglo de enzimas capaces de biotransformar una amplia variedad de compuestos con diferentes funciones y estructuras químicas.

La detoxificación enzimática de xenobióticos se ha clasificado en tres diferentes fases. Las fases I y II involucran la conversión de un compuesto lipofílico, un xenobiótico no polar, a un metabolito más soluble y menos tóxico que es eliminado en la fase III (Sheehan *et al.*, 2001). La fase I es catalizada principalmente por el sistema del citocromo P450. Las enzimas de la fase II catalizan la conjugación de los xenobióticos activados a un substrato endógeno, soluble en agua, como el glutatión reducido (GSH), ácido UDP glucurónico o glicina. Cuantitativamente, la conjugación con GSH que es catalizada por las GSTs es la principal reacción de la fase II en muchas especies (Sheehan *et al.*, 2001). Para la eliminación de compuestos tóxicos, o xenobióticos, existe un grupo de aproximadamente 30 enzimas llamadas "enzimas de detoxificación" (Jakoby y Ziegler, 1990), en donde se incluye a la glutatión transferasa (GST).

Glutatión S-Transferasas

Las GSTs realizan una gran variedad de funciones en las células, tales como la remoción de especies reactivas del oxígeno y la regeneración de proteínas con grupos tiol, ambos consecuencia del estrés oxidativo, conjugaciones con ligandos endógenos además de catálisis de reacciones en rutas metabólicas no asociadas con la

detoxificación. Sin embargo, han sido más estudiadas por su importante participación en los procesos de detoxificación. Las GSTs son un grupo de enzimas que catalizan la conjugación del glutatión (GSH) con una gran variedad de electrófilos, así como la reducción de oxidantes por medio del GSH. Ésta actúa sobre un gran número de compuestos xenobióticos y por lo general cataliza el paso inicial en los procesos de detoxificación (Denison y Whitlock, 1995). Las GSTs se encuentran ampliamente distribuidas en los seres vivos, donde su papel principal es el mantenimiento de la integridad celular.

Las GSTs son enzimas citosólicas que provienen de una familia de multigenes que pueden ser expresadas constitutivamente o ser inducidas por un gran número de compuestos, tanto de origen natural como xenobióticos (Vuilleumier, 1997). Además son diferencialmente reguladas, expresándose enzimas diferentes en diversos tejidos durante cada estado de desarrollo y en respuesta a varios xenobióticos (Ranson *et al.*, 1997). Las GSTs están implicadas en el desarrollo de la resistencia a fármacos, pesticidas, herbicidas y antibióticos de un gran número de organismos. Un incremento en los niveles de expresión de GST se ha correlacionado con la resistencia a los insecticidas, como el DDT utilizado en el control de la malaria que ha propiciado la aparición de especies resistentes del mosquito *Anopheles* (Ranson *et al.*, 1997), y principalmente a compuestos organofosforados en varios insectos (Kostaropoulos *et al.*, 2001; Rosa de Lima *et al.*, 2002).

Algunos tipos de GSTs tienen una alta especificidad catalítica por epóxidos carcinógenos y pesticidas, de tal manera que la ausencia, presencia o modulación de estas isoenzimas puede afectar marcadamente la susceptibilidad de especies particulares a algunos tóxicos químicos (Henson *et al.*, 2000). En invertebrados marinos se ha estudiado la actividad de GST como un biomarcador de la exposición a este tipo de tóxicos, en crustáceos como el cangrejo *Carcinus maenas* (Gowlan *et al.*, 2002) y en el molusco *Mytilus edulis* (Astley *et al.*, 1999). Hasta la fecha, son escasos los estudios de este tipo en camarón, siendo un organismo de gran importancia económica. En este trabajo se purificó una GST clase mu a partir de branquias de camarón (Contreras-

Vergara *et al.*, 2007), que mostró altos valores de actividad que podrían relacionarse con un mecanismo de detoxificación muy eficiente en las branquias, y que puede ser el resultado de la necesidad de una respuesta eficiente a un posible ambiente contaminado, ya que se encuentran expuestas de manera directa.

GSTs: Versatilidad funcional dentro de un esquema conservado

Clasificación de las GSTs

A las GSTs (EC 2.5.1.18) también se les llama Glutatión S-alquiltransferasa, Glutatión S-ariltransferasa, S-(hidroxialquil)glutatión liasa y Glutatión S-aralquiltransferasa; de acuerdo al radical que transfieran al glutatión (IUBMB) (http://www.expasy.ch/cgi-bin/nicezyme.pl2.5.1.18).

Existen diferentes familias de GSTs, a las cuales se les ha llamado clases. Aún no está claramente establecido un criterio para colocar a una GST en una clase particular; pero generalmente es aceptado que las GSTs que comparten más del 60% de identidad se encuentran dentro de una misma clase y aquellas con menos del 30% de identidad son asignadas a clases diferentes. Se ha puesto énfasis particular en el extremo N-terminal de la estructura primaria, ya que dentro de una clase, esta región tiende a ser más conservada y en ella se encuentran residuos catalíticamente esenciales que forman parte del sitio activo. Existen también otros criterios que se utilizan para la clasificación de las GSTs, tales como técnicas de reconocimiento por anticuerpos específicos (Hayes y Mantle, 1986), propiedades cinéticas (Mannervik *et al.*, 1985), pruebas de complementación entre subunidades, así como el estudio de la estructura cristalina y especificidad por sustratos modelo (como el 1-cloro-2-4-dinitrobenceno o CDNB).

En base a una combinación de los criterios anteriores, las GSTs citosólicas de mamíferos fueron clasificadas primeramente en las clases alfa (α), mu (μ) y pi (π), que han sido estudiadas principalmente en rata, ratón y humanos. La hipótesis de que las

clases representan familias separadas de GSTs, es apoyada por las distintas estructuras de sus genes y su localización cromosomal. Los genes de las clases alfa, mu y pi difieren marcadamente entre ellas en tamaño y estructura intrón/exón (Hayes y Pulford, 1995). Dentro de una clase particular se pueden identificar subfamilias claramente definidas (subclases). Cada subfamilia puede incluir hasta 5 enzimas diferentes, las cuales pueden compartir hasta un 90% de identidad. Hasta ahora se conocen al menos, 6 distintas subclases de la clase mu en rata (M1, M2, M3, M4, M5 y M6) con sus homólogos para los primeros 5 en humanos así como 6 GSTs clase alfa (A1, A2, A3, A4, A5 y A6) en rata, con sus homólogos para los primeros 4 en humanos (Eaton y Bammler, 1999).

Además de las clases mencionadas anteriormente, se han descrito otras GSTs clasificadas como teta (θ) y zeta (ζ) en humanos (Board *et al.*, 1997) y la clase kappa (κ) en rata, humano (Pemble *et al.*, 1996) y en ratón (Jowsey *et al.*, 2003). Este sistema de clasificación, desarrollado originalmente para las GSTs de mamíferos, se ha extendido a GSTs de otros organismos en los que a su vez se han descubierto nuevas clases, que después se encontraron también en mamíferos. Así, se conocen ahora las clases beta (β), lambda (λ), sigma (σ), omega (ω) y tao (τ). En cefalópodos y artrópodos también se ha encontrado GST de la clase sigma (σ); en plantas se ha descrito una clase phi (Φ) y en insectos una clase delta (δ), la cual anteriormente era referida como teta (Ketterman *et al.*, 2001). Las formas más conocidas de las GSTs son citosólicas, sin embargo también se ha descubierto una GST microsomal que se encuentra unida a la membrana celular (Jakobsson *et al.*, 1997; Sun y Morgenstern, 1997).

Características estructurales

La versatilidad funcional y la variabilidad de las secuencias de las GSTs, puede entenderse en base a la conservación de esquemas estructurales a través de las diferentes clases de GSTs. Las diferentes clases de GSTs son divergentes, exhibiendo sólo entre el 20 y 30 % de identidad a través de las enzimas de mamíferos más

similares estructuralmente, las clases alfa, mu y pi. A pesar de la baja similitud a nivel de secuencia entre las clases, estructuralmente todas comparten una forma característica. En general, las GSTs tienen una estructura homodimérica, en donde cada monómero (de alrededor de 220 aminoácidos y 25 kDa) está compuesto de dos dominios, el N-terminal y el C-terminal.

En el dominio N-terminal (aproximadamente residuos 1- 80) se encuentra el sitio de unión al glutatión (sitio G) que consiste en un módulo $\beta\alpha\beta\alpha\beta\beta\alpha$. Este dominio tiene un plegamiento tipo tioredoxina (Martin, 1995), en donde se encuentran los principales determinantes para la unión al GSH. El lazo que conecta la hélice $\alpha 2$ con la hoja $\beta 3$ tiene un residuo de prolina característico que es muy conservado en todas las GSTs. Este residuo no participa directamente en la catálisis, pero parece ser importante en el mantenimiento de la estructura de la proteína para que sea catalíticamente competente (Sheehan *et al.*, 2001).

El dominio C-terminal, llamado también sitio H (aproximadamente residuos 87 – 210), tiene una estructura predominantemente hélice α, que varía en número de hélices entre las diferentes clases. Las clases mu y pi presentan 5 hélices (Ji *et al.*, 1992), mientras que en la clase alfa se observan seis (Sinning *et al.*, 1993). Este dominio, que es más variable que el N-terminal entre las clases de GSTs, provee los principales elementos estructurales asociados con la especificidad hacia el segundo sustrato, relacionándose con la unión a sustratos hidrofóbicos de una manera menos específica.

Además de las similitudes, existen algunas características estructurales específicas de cada clase, tales como el llamado lazo mu, común en todas las enzimas clase mu, y la presencia de una hélice alfa extra, la hélice $\alpha 9$, común en todas las GSTs clase alfa (Figura 3). Se ha sugerido que la localización de estos elementos estructurales adyacentes a los sitios de unión del substrato contribuyen a la formación de una arquitectura del sitio activo de alguna forma restringida para las clases alfa y mu, comparadas con las clases pi, sigma y tao (Babbitt, 2000). Estudios recientes en una GST M1-1 de rata indican que el lazo mu no es esencial para mantener la

estructura, ni es requerido para que la enzima mantenga su actividad catalítica; aunque si es una determinante para la afinidad de la enzima por los sustratos (Hearne y Colman, 2006a).

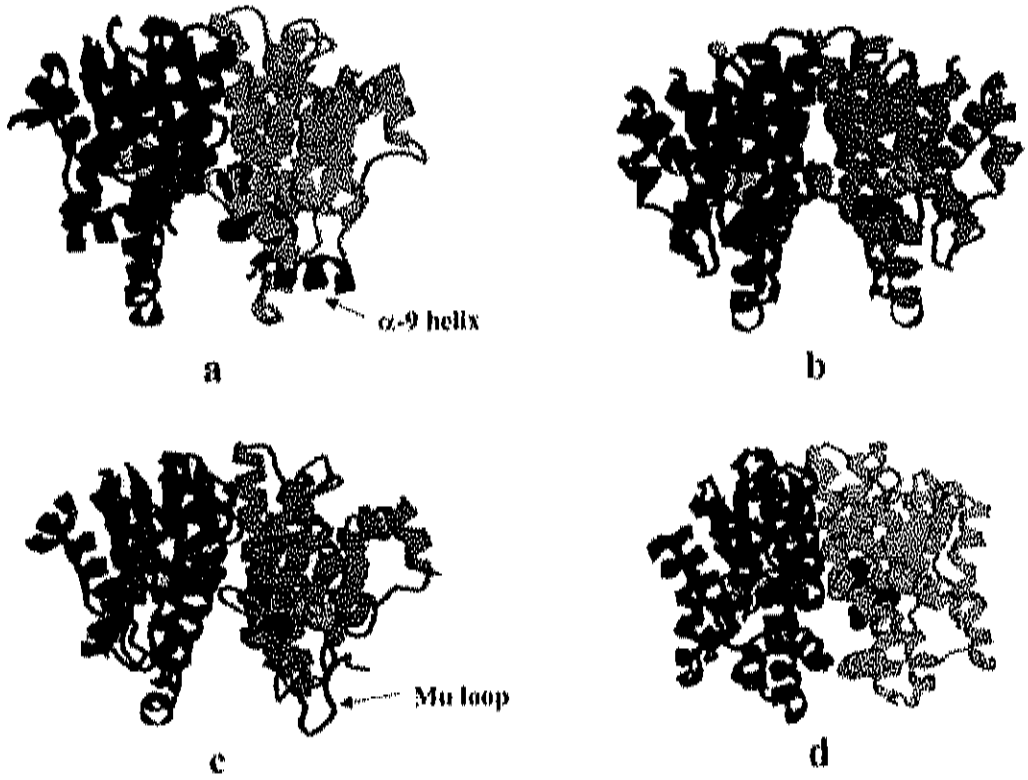


Figura 3. Estructuras de GST. Arreglos estructurales para diferentes tipos de GSTs. a) clase alfa humana (1GUH), b) clase pi humana (1GSS), c) clase mu de rata (6GST), d) clase teta humana (1LJR). (Fuente: (Sheehan *et al.*, 2001)

La interfase del dímero es de aproximadamente 25 Å X 35 Å; a una altura de 25 Å se separa para crear un hueco en forma de V, al cual es accesible el solvente (Sinning *et al.*, 1993). En esta interfase existen interacciones predominantemente hidrofóbicas que se forman entre residuos del dominio N-terminal de un monómero y el dominio C-terminal del otro monómero. La única interacción entre el sitio activo de una

subunidad y la otra, es un residuo de ácido aspártico (en la hélice α4), que contribuye en el sitio de unión al glutatión. En un estudio de Hearne y Colman (Hearne y Colman, 2006b), encontraron que no se requiere la formación del dímero para que la enzima cumpla con su función catalítica, aunque el dímero resulta más activo. En las clases alfa, mu y pi, se encuentra de manera conservada, un residuo de tirosina en el dominio N-terminal (en la hoja β1), el cual se encuentra a un puente de hidrógeno de distancia del grupo tiol del GSH, y se ha demostrado que es esencial durante la catálisis (Armstrong, 1997).

Mecanismo catalítico

Como grupo, las GSTs muestran una amplia versatilidad catalítica, debido a su variabilidad del dominio C-terminal que le confiere características específicas a cada clase de GST, e incluso a cada subclase. De esta manera, las GSTs reaccionan mediante un ataque nucleofílico, casi con cualquier compuesto hidrofóbico que contenga un átomo electrofílico, tales como carbono (C), nitrógeno (N), azufre (S) y oxígeno (O): Ataque sobre C: Donde las GSTs actúan como S-alquiltransferasa, S-ariltransferasa, S-epoxidotransferasa o S-alquenotransferasa, de acuerdo al radical que transfieren; Ataque sobre N: En ésteres orgánicos de nitrato; Ataque sobre S: En tiocianatos orgánicos y disulfuros y Ataque sobre O: En peróxidos orgánicos.

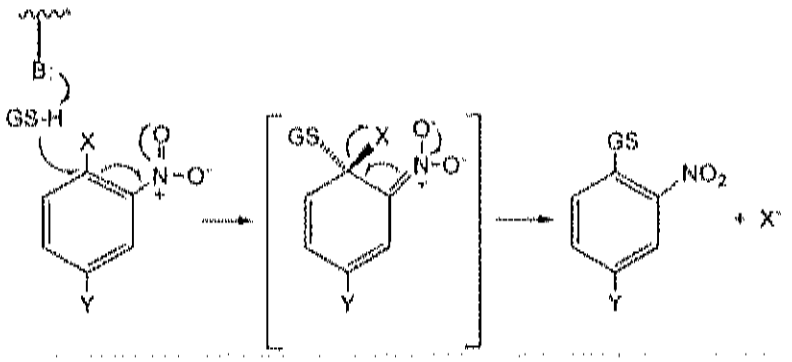
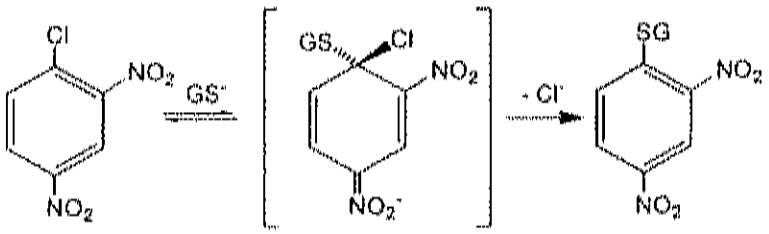


Figura 4. Figura de la reacción general de una sustitución nucleofílica aromática (S_NAr) por el glutatión. (Fuente: Silverman, 2000)

Las GSTs catalizan la adición nucleofílica del tripéptido glutatión (GSH) a compuestos electrófilos por medio de mecanismos S_N2, adición de Michael o S_NAr (Figura 4) (Silverman, 2000).

Uno de los substratos mas utilizados en el estudio del mecanismo de acción de esta enzima es el 1-cloro-2-4-dinitrobenceno (CDNB), en el que la GST cataliza una substitución nucleofílica en el compuesto aromático mediante una dehalogenación (S_NAr) (figura 5). En esta reacción el grupo tiol (-S) del glutatión actúa sobre el carbono del anillo aromático del CDNB, el cual tiene como substiyuente al cloro, formando un compuesto intermediario llamado "complejo Meisenheimer", que después de sufrir una dehalogenación (en este caso la eliminación del cloro), se transforma en el producto conjugado 1-(S-glutationil)-2,4-dinitrobenceno (GSDNB) (Figura 5). Durante la reacción, los residuos que se encuentran en el sitio activo de la enzima participan estabilizando al complejo formado por el GSH y el sustrato hidrofóbico, así como también brindando asistencia electrofílica en la catálisis.



CDNB | Complejo de Meisenheimer | GSDNB

Figura 5. Formación del producto conjugado de glutatión y el sustrato CDNB. (Fuente: Johnson *et al.*, 1993)

Estudios cristalográficos realizados en diferentes GSTs, han revelado los residuos que forman el bolsillo hidrofóbico en donde se acomoda el sustrato en el sitio

activo. En una GST clase mu de rata (isoenzima 3-3), los residuos que se encuentran en la región del sustrato hidrofóbico incluyen a la Y6, W7, V9, L12, I111, Y115, F208 y S209 (Ji *et al.*, 1994), los cuales en su mayoría se encuentran en contacto con el sustrato por medio de interacciones de van der Waals. Por otro lado, se sabe que el grupo tiol del GSH unido a la enzima es estabilizado por un puente de hidrógeno con un residuo de tirosina (en las clases mu y pi), una serina (clase teta) o arginina (clase alfa). Para conocer la forma en que estos residuos interactúan con el sustrato durante la catálisis, se han realizado estudios con mutantes obtenidas por medio de mutagénesis sitio-dirigidas que han sido analizadas por medio de cristalografía.

Mutantes dirigidos de GST: Estudios de estructura y función

Aunque todas las GSTs comparten ciertas características estructurales generales, existen variaciones en sus secuencias que tienen efectos significativos en las propiedades de las enzimas, de tal forma que variaciones de sólo 1 ó 2 aminoácidos pueden ocasionar un cambio en la estructura terciaria de la proteína, lo cual se traduce en cambios importantes en la actividad de la enzima (Ketterman *et al.*, 2001). Estudios por medio de mutaciones dirigidas a residuos específicos han revelado la importancia de estos residuos en el sitio de unión con el glutatión y con el sustrato hidrofóbico. Las GSTs más estudiadas son las de rata y humano, de las cuales existe una gran cantidad de estructuras reportadas y que además, es en las que más se ha investigado el efecto de estos cambios. Dada la variedad de GSTs reportadas, se dará especial énfasis a la clase mu, que es el objeto de este estudio.

En el sitio de unión al glutatión de las GSTs clase mu se encuentra, de manera muy conservada, un residuo de tirosina (Y6 en rata) localizado a un puente de hidrógeno de distancia del grupo tiol del glutatión. Este residuo ha sido reemplazado por diferentes aminoácidos en las GSTs M1-1 y M3-3 de rata y en A1-1 y P1-1 de humano; en todos los casos, la mutación resultó en una disminución de la actividad específica de la GST. Estos resultados, junto con los datos cristalográficos, mostraron que la Y6

desempeña una función esencial en la estabilización del tiol a través de un puente de hidrógeno (Rushmore y Pickett, 1993). Este residuo puede estar substituido por una serina (clase teta) o una arginina (clase alfa). Esta interacción ha sugerido ser la responsable, en gran parte, del bajo valor de pK_a del GSH en el complejo enzima-GSH. La eliminación de esta interacción en una mutante Y6F de M1-1 de rata (Liu *et al.*, 1993) afecta la eficiencia catalítica de la enzima y aumenta el pK_a del tiol unido a la enzima. Lo anterior indica que una importante contribución de la enzima en la catálisis es la disminución del pK_a del GSH unido a ella.

Por medio del uso de calorimetría de titulación isotérmica (ITC) se han realizado algunos estudios con mutantes Y7F de GST del nemátodo *Schistosoma japonicum*, donde se ha observado una disminución en la actividad de la enzima mutante hacia el substrato CDNB, así como un aumento en al afinidad hacia el GSH, comparado con la enzima nativa (Andújar-Sánchez *et al.*, 2003; Yassin *et al.*, 2003).

De los residuos implicados en el sitio de unión del sustrato hidrofóbico, la Y115 (GST de rata) se ha confirmado que participa de una manera importante en la catálisis de las GSTs clase mu. Este residuo se localiza al final de la hélice α4 y cerca del sitio activo, influyendo en la eficiencia catalítica de la enzima (Shan y Armstrong, 1994). En una mutante Y115F, la remoción del grupo hidroxilo de la tirosina tuvo un efecto muy marcado en la catálisis de la adición del GSH a un óxido de fenantreno, reduciendo k_{cat} y k_{cat}/K_m en 2 órdenes de magnitud, indicando una interacción crucial para la reacción química (Johnson *et al.*, 1993). Sin embargo, utilizando CDNB como sustrato, la mutante Y115F es más eficiente que la nativa, lo cual demuestra que el grupo hidroxilo de la enzima nativa no provee un beneficio catalítico en esta reacción. La Y115 se encuentra hacia el extremo terminal carboxilo y forma parte del sitio H o sitio de unión con el xenobiótico. En la GST clase mu de rata, este residuo participa en el mecanismo catalítico, ya que provee asistencia electrofílica en la adición del GSH a un sustrato (Johnson *et al.*, 1993). En rata, la mutante Y115F de una GST tipo mu ha demostrado ser un mejor catalizador que la proteína nativa hacia el CDNB.

Glutatión S-Transferasa clase mu de *L. vannamei*

En un trabajo anterior (Contreras-Vergara *et al.*, 2004), se encontró una secuencia nucleotídica de 852 pb (con un ORF de 657 pb) similar a Glutatión S-Transferasa (GST) tipo mu, en el camarón *Litopenaeus vannamei*. Esta secuencia mostró valores de identidad a nivel de aminoácidos, de 56% con GST de rata *Rattus norvegicus* (P04905) y 55% con GST de ratón *Mus musculus* (P15626) y de humano (P46439) (Figura 6). Estas similitudes se encuentran dentro de los valores que se han reportado entre secuencias de proteínas de GSTs dentro de una misma clase y son mayores al 50% (Vuilleumier, 1997; Ketterman *et al.*, 2001). La secuencia nucleotídica codifica para una proteína de 219 aminoácidos, que corresponde a un monómero de la proteína GST, con un peso molecular calculado de 25.519 kDa y un punto isoeléctrico (pI) de 6.89 (Contreras-Vergara *et al.*, 2004). Se han encontrado pesos moleculares muy similares en GSTs de otros organismos (Ranson *et al.*, 1997; Rosa de Lima *et al.*, 2002).

Una de las características de las GSTs es que la región del N-terminal es altamente conservada; es en esta región donde se encuentra el sitio de unión al glutatión (Vuilleumier, 1997; Ketterman *et al.*, 2001). Como en otras GSTs, la GST de camarón contiene la región altamente conservada FPNLPYYIDGD entre los residuos 58 y 67 (Contreras-Vergara *et al.*, 2004), que corresponde al sitio G de unión al glutatión (Rosa de Lima *et al.*, 2002).

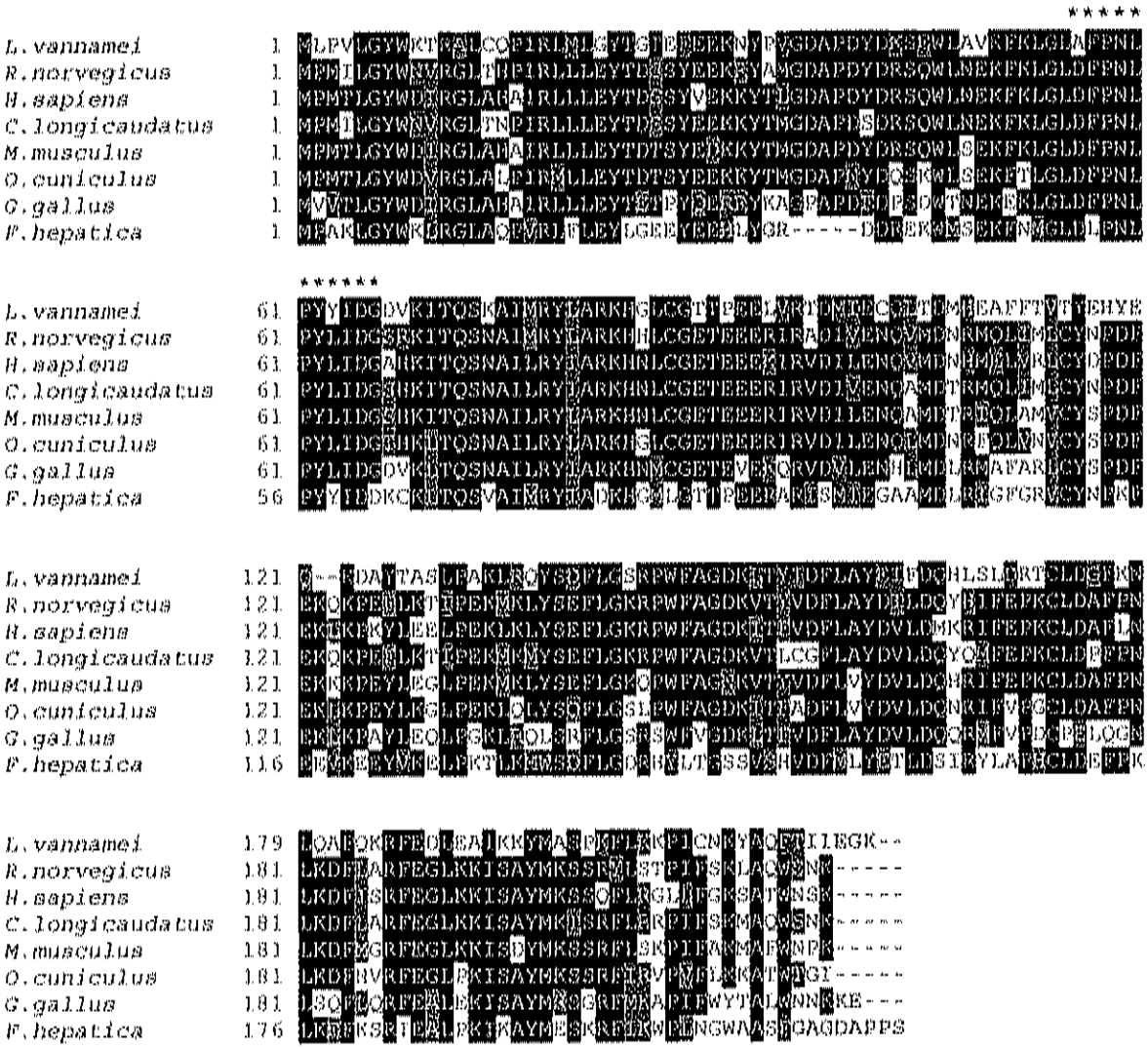


Figura 6. Alineamiento de secuencias aminoacídicas entre GST de *L. vannamei* y otros organismos. La región marcada con asteriscos corresponde al sitio de unión con el GSH. Las regiones sombreadas en negro, representan a los aminoácidos idénticos de GST de varios organismos y las sombreadas en gris indican los reemplazos conservados.

En este trabajo se obtuvo un modelo de la estructura terciaria de la GST clase mu de camarón (Figura 7). Esta modelación realizada por homología con la estructura de una GST clase mu de humano (hGSTM1-1) obtenida por análisis cristalográfico, muestra que se mantiene la topología del plegamiento observada en otras GSTs de diferentes organismos, en particular de la clase mu. Sin embargo se encontraron

pequeñas diferencias en el sitio activo, que se ven reflejados en los valores de las constantes cinéticas para la enzima silvestre recombinante, donde se observó un aumento en la velocidad de catálisis de un orden de magnitud, comparada con la de humano.

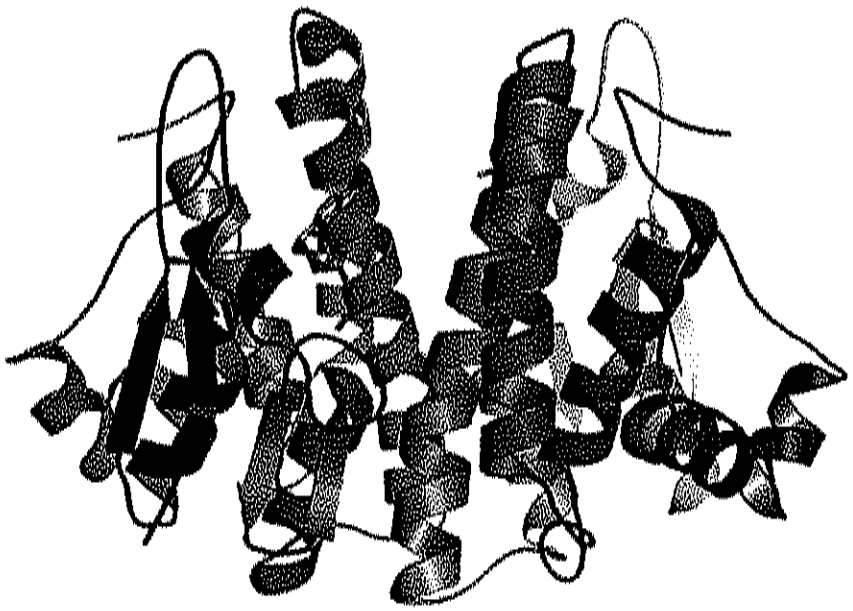


Figura 7. Representación de lazos del modelo teórico de la GST clase mu de camarón *L. vannamei*.

El valor de velocidad de catálisis (k_{cat}) para la enzima silvestre recombinante de camarón resultó muy similar al reportado para la GST clase mu purificada a partir de branquias (Contreras-Vergara *et al.*, 2007). Sin embargo, la eficiencia catalítica (k_{cat}/K_m^{GSH}) resultó mayor para la enzima recombinante, esto debido a la mayor afinidad (K_m) de esta enzima por el sustrato GSH. Aún así, en la GST purificada de branquias se observaron valores altos de actividad específica, aún mayores que los reportados para otros invertebrados marinos, por lo cual se podría pensar en un mecanismo de detoxificación muy eficiente en las branquias del camarón.

En las mutantes Y7F y Y116F de la GST clase mu de camarón, obtenidos en este trabajo, se conservan las mismas interacciones existentes en el sitio activo de la WT: puentes de hidrógeno con W46, N59, L60, S73, Q72 e interacciones de van der Waals con L13, Y7, F112, además de una interacción con el residuo de D106 proveniente de la otra subunidad. En estas mutantes no se observaron cambios importantes en las constantes catalíticas que reflejaran algunos cambios en el sitio activo, sobre todo en las interacciones entre residuos importantes para la catálisis. La mutante Y116 es la que presentó mayor cambio. Aunque no afectó de manera importante a la topología del sitio activo, el efecto se reflejó en los valores de las constantes cinéticas, presentando un ligero aumento en la velocidad de catálisis con respecto a la GST silvestre, lo cual finalmente resultó en una disminución en la eficiencia catalítica, debido a la menor afinidad hacia los sustratos. Por otra parte, también se observó la presencia de otros residuos, que se encuentran de manera poco común en las GSTs de esta clase, que pudieran intervenir de forma importante en la catálisis de esta enzima, por lo cual tal vez las mutaciones Y7F y Y116F no reflejan cambios importantes en las constantes cinéticas.

De esta manera podemos decir que las pequeñas diferencias de las constantes cinéticas de estado estable de Michaelis-Menten, pueden sugerir diferencias estructurales que modifican la catálisis. Esto es, que la relación estructura-función sugiere que la pérdida de capacidad catalítica, ya sea en reducción de la eficiencia catalítica o la reducción en afinidad por los substratos, indican un arreglo estructural sub-óptimo para la realización de la catálisis.

PROPÓSITO DE LA TESIS

El camarón blanco *Litopenaeus vannamei* (camarón blanco del occidente) es uno de los crustáceos marinos más importantes debido a su alta demanda comercial y a su relativa facilidad de cultivo comparada con otros camarones peneidos. Esto es debido a que *L. vannamei* soporta altas densidades de población en estanque y sus requerimientos proteínicos son menores que los de otras especies. Sin embargo, también puede encontrarse en condiciones ambientales no favorables, como puede ser la localización de las granjas, en donde pueden estar expuestos a compuestos xenobióticos, provocando un estrés oxidativo que genera compuestos tóxicos adicionales a los producidos durante el metabolismo normal (Dandapat *et al.*, 2000).

Los sistemas de detoxificación realizan una función muy importante, ya que protegen del estrés oxidativo a todos los organismos. La mayoría de los estudios sobre estos sistemas se han enfocado a insectos en donde la resistencia a ciertos compuestos xenobióticos es mediada por la inducción de enzimas, tales como la Glutatión S-Transferasa. Para contribuir al conocimiento en ésta área en un organismo tan importante económicamente como el camarón, en este trabajo se purificó una GST clase mu a partir de branquias de camarón blanco, la cual posteriormente se caracterizó cinéticamente. Estos resultados se muestran en el capítulo 2.

Para profundizar en la importancia de residuos de aminoácidos específicos en la catálisis de esta enzima, se realizaron cambios por medio de mutagénesis sitio-dirigida en dos de estos residuos. De esta manera, se consideró la información generada a partir de un modelo por homología y se probó experimentalmente el efecto de las mutaciones sobre las características cinéticas de la GST clase mu de camarón blanco. El capítulo 3 se enfoca en la caracterización bioquímica de la GST clase mu silvestre y sus mutantes Y7F y Y116F, producidas de forma recombinante en *E. coli* y basado en su cDNA aislado anteriormente (Contreras-Vergara *et al.*, 2004) (Anexo).

HIPÓTESIS

Las mutaciones Y7F y Y116F de la GST clase mu modifican la capacidad catalítica de la enzima, sin necesariamente disminuir la afinidad de la enzima por los substratos.

OBJETIVOS

OBJETIVO GENERAL

Caracterizar las formas nativa y mutantes de una enzima GST clase mu de camarón blanco *Litopenaeus vannamei*.

OBJETIVOS PARTICULARES

1. Determinar las características cinéticas de la GST nativa de branquias del camarón.

2. Obtener mutantes de la GST clase mu de camarón blanco utilizando sobreexpresión recombinante.

3. Investigar el efecto de mutaciones puntuales dirigidas sobre las características cinéticas (V_{max} y K_m) de la GST.

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2

A Mu Class Glutathione S-Transferase From Gills of the Marine Shrimp *Litopenaeus vannamei*: Purification and Characterization

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A Mu-Class Glutathione *S*-Transferase From Gills of the Marine Shrimp *Litopenaeus vannamei*: Purification and Characterization

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ABSTRACT: Glutathione *S*-transferases (GSTs) are a family of detoxifying enzymes that catalyze the conjugation of glutathione (GSH) to electrophiles, thereby increasing the solubility of GSH and aiding its excretion from the cell. In this study, a glutathione *S*-transferase from the gills of the marine shrimp *Litopenaeus vannamei* was purified by affinity chromatography using a glutathione–agarose affinity column. GST was purified to homogeneity as judged by reducing SDS-PAGE and zymograms. This enzyme is a homodimer composed of ~25-kDa subunits and identified as a Mu-class GST based on its activity against 1-chloro-2,4-dinitrobenzene (CDNB) and internal peptide sequence. The specific activity of purified GST was 440.12 μmol/(min mg), and the K_m values for CDNB and GSH are very similar (390 and 335 μM, respectively). The intersecting pattern of the initial velocities of this enzyme in the Lineweaver–Burke plot is consistent with a sequential steady-state kinetic mechanism. The high specific activity of shrimp GST may be related to a highly effective detoxification mechanism necessary in gills since they are exposed to the external and frequently contaminated environment. J Biochem Mol Toxicol 21:62–67, 2007; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10:1002/jbt.20162

KEYWORDS: Purification; Gills; Glutathione *S*-Transferase; Shrimp; *Litopenaeus vannamei*; Kinetic Characterization

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INTRODUCTION

Glutathione *S*-transferases (GST; E.C. 2.5.1.18) are multifunctional dimeric phase II enzymes that are capable of detoxifying endogenous and exogenous substances by conjugation to glutathione, converting a reactive lipophilic molecule into a water-soluble (nonreactive) conjugate to be excreted [1]. GSTs have been found in virtually all organisms studied, including plants, animals, and bacteria. Eukaryotes usually contain multiple GSTs with different catalytic abilities to accommodate a wide range of functions within the cells [2]. Cytosolic GSTs are dimers composed of subunits of ~25 kDa [2] that contain a GSH-binding site (the G site) and a hydrophobic substrate-binding site (the H site) in each subunit. While the G site is well conserved among GSTs, the H site varies widely in different classes, leading to differences in substrate selectivity [3]. Mammalian cytosolic GSTs are grouped into different classes, based primarily on protein sequence similarity and substrate specificity and named Alpha, Mu, Pi, Theta, Zeta, Sigma, and Omega [2,3]; additionally, the Kappa GST is a soluble mitochondrial enzyme. New classes of GSTs have been found in nonmammalian species, such as the Beta class found in bacteria, the Phi and Tau classes of plants, as well as the Delta class described in insects [4].

GST isoenzymes have different subunit structure, isoelectric points, kinetics, and immunological properties. Marine invertebrate GSTs are not as well known as vertebrate GSTs, even though reports about their induction after exposure to toxic chemicals are available. GSTs are amply used as biomarkers in bivalve molluscs like the blue mussel *Mytilus edulis* [5], the brown mussel *Perna perna* [6], and the clam *Ruditapes decussatus* [7];

and in crustaceans like the water flea *Daphnia magna* [8], the crayfish *Procambarus clarkii* [9], and the freshwater crustacean (scud) *Gammarus italicus* [10]. GST activity was induced in the water flea *Daphnia magna* after exposure to phenobarbital [11] and in the crab *Carcinus maenas* after treatment with cypermethrin [12]. Likewise, in the abalone *Haliotis rufescens* and black chiton *Katharina tunicate*, GST activity increased upon treatment with a synthetic xenobiotic [13]. Several GST isoforms were described in the clam *Ruditapes decussatus*; interestingly, the N-terminus of one of the subunits has sequence similarity to Alpha, Mu, and Pi GSTs from the parasite (liver fluke) *Fasciola hepatica* [14]. In the shrimp *Penaeus japonicus*, two anionic GSTs were purified from the eyes. One of these GSTs was identified as Mu and the other as Theta, based on the sequence of their N-terminus [15].

Invertebrate GSTs appear to be also expressed in several tissues and different developmental stages, although they have not been studied as much as their vertebrate counterparts [16]. However, little is known about their sequence, expression, and structure. In previous work [17], we reported a cDNA sequence for a Mu-class shrimp GST and detected its transcript in hepatopancreas, hemocytes, gills, and muscle. The shrimp GST sequence was computer modeled and found to fit the classical two-domain GST structure, where Domain I, which contains the glutathione (GSH)-binding site, is more conserved compared with the flexible C-terminal Domain II. In this article, we present the purification and kinetic characterization of a GST from the gills of the shrimp *Litopenaeus vannamei*.

MATERIALS AND METHODS

Enzyme Purification

Gills from white shrimp *Litopenaeus vannamei* were dissected and stored at −80°C until used. They were homogenized at 4°C with a mortar and pestle in 4 volumes of cold PBS, pH 7.3, containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 5 mM DTT, and 1 mM PMSF (Buffer A). The homogenate was centrifuged at 22,000 × *g* for 30 min, 4°C, and the supernatant (gills extract) was centrifuged again at 45,000 × *g* for 8 h to eliminate hemocyanin (the shrimp oxygen carrier protein). The clear supernatant (clarified gills extract) was used for affinity chromatography using a GSH–agarose column (Amersham Biosciences). The clarified gills extract was applied to the column, equilibrated with buffer A, and the protein bound to the column was eluted with 50 mM Tris–HCl, pH 8.0, containing 10 mM GSH and 5 mM DTT (Buffer B) using an ÄKTA purifier (Amersham). Fractions (1.5 mL) with protein and GST activity were collected, pooled, and concentrated by diafiltration using YM10 membrane (Millipore). The buffer was exchanged with Buffer A containing 10% glycerol; the purified GST was frozen at −20°C and used for electrophoretic and kinetics studies.

Determination of Protein Concentration and GST Activity

Protein concentration of the fractions was determined by the method of Bradford [18]. GST activity was measured in a Cary 50 Bio UV–visible spectrophotometer (Varian) at 25°C using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [19]. The reaction mixture contained 100 mM Na_2HPO_4, pH 6.5, and 1 mM each of GSH and CDNB in a final volume of 1 mL. Each determination was performed in duplicate. Rates in the absence of substrates were negligible. One unit of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per minute under the conditions described above. Specific activity was expressed as micromoles of product per minute per milligram of protein, and GST activity was calculated using the extinction coefficient $E = 9.6\ mM^{-1}\ cm^{-1}$ [19].

Polyacrylamide Gel Electrophoresis (PAGE/SDS-PAGE) and Zymograms

Reducing 10%–20% polyacrylamide gradient gels (SDS-PAGE) was done using a BioRad Mini Protean III electrophoresis unit. Gels were stained with Coomassie brilliant blue [20] or silver stain [21]. GST activity was detected in 9% native PAGE [22]. After electrophoresis, the gel was incubated in 0.1 M potassium phosphate, pH 6.5, 4.5 mM reduced glutathione, 1 mM CDNB, and 1 mM NBT (nitroblue tetrazolium) for 10 min under gentle agitation. Then, the gel was washed with water and incubated in 0.1 M Tris–HCl, pH 9.6, containing 3 mM phenazine methosulfate until achromatic bands (activity bands) appeared on the dark-blue-stained gel. The reaction was stopped by rinsing the gel with water.

Internal Sequence Determination

The purified GST was subjected to in-gel trypsin digestion, and an internal peptide sequence was obtained by nanospray liquid chromatography–tandem mass spectrometry (LC-MS/MS) at the Centre Hospitalier de l'Université Laval (CHUL) Research Centre of Eastern Quebec Proteomics Center. Sample preparation, SDS-PAGE, and staining of the gel for sequence determination were carried out according to the recommendations of the CHUL Research Centre.

Enzyme Kinetics

Kinetic parameters were determined for GSH and CDNB substrates by recording the activity toward a range of concentrations from 0.1 to 1.6 mM for both substrates, varying the concentrations of one substrate while keeping the other constant. Experimental data at a fixed concentration of one substrate and varying concentration of the second were fitted to the Michaelis–Menten equation, by using the nonlinear regression analysis program GraFit (Erithacus Software).

RESULTS

GST Purification and Activity

A summary of the purification steps used for *L. vannamei* GST is presented in Table 1. The affinity-purified GST-specific activity toward CDNB was 440.12 μmol/(min mg), when the enzyme was purified 106-fold. Analysis of the fractions by reducing SDS-PAGE revealed a purified protein of ~25 kDa (Figure 1, panel A) and ~50 kDa in native conditions and in the zymogram gel (Figure 1, panel B), indicating purity and homogeneity. Therefore, the shrimp GST is a homodimer, with sizes similar to most known GSTs [2]. A band of ~240 kDa was seen in the native gels and correspond to aggregates of the enzyme, since only the 25-kDa band was detected via SDS-PAGE. Achromatic bands may also result in zymograms from superoxide dismutase activity, but the 50- and 240-kDa bands were detected only in the presence of CDNB, confirming GST activity (data not shown). After in-gel digestion of the pure native GST and mass spectrometry, an internal peptide with the sequence KVTQSNAILRY was obtained. Comparison of this sequence to protein database identified as the closest matching amino acids ^{69}KITQSKAIMRY^{79} from the previously deposited *L. vannamei* GST [17], followed by ^{69}KITQSNAIMRY^{79} from rat Mu GST (Accession number 38648907). The slight differences in amino acids between the cDNA deduced sequence and the determined peptide sequence may be due to polymorphism between shrimp. Therefore, we conclude that the purified native protein corresponds to the cDNA deduced amino acid sequence previously isolated from the *L. vannamei* hepatopancreas library [17].

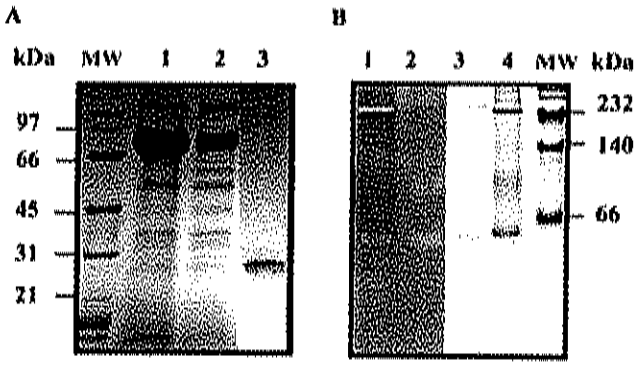

FIGURE 1. Purification of gills GST. Panel A: SDS-PAGE; lane 1; gills extract; lane 2, clarified gills extract; lane 3, purified GST; MW markers (BioRad). Panel B: zymogram and native gel; lanes 1 and 2 show zymogram for purified GST and clarified homogenate, respectively; lanes 3 and 4 show Coomassie-stained native gel for purified GST and clarified homogenate, respectively; MW, markers (BioRad).

Kinetic Studies

The plots of initial velocity (v) versus [GSH] or v versus [CDNB] in the range 0.1–2 mM GSH and 0.1–1.6 mM CDNB followed Michaelis–Menten kinetics (not shown). The corresponding families of double-reciprocal plots were linear and gave normal intersecting patterns (Figures 2 and 3). This information determines the type of kinetic mechanism of the enzymatic reaction, and our data indicate that the mechanism of the *L. vannamei* GST is not ping-pong type and should be sequential, either a Steady State Ordered, Steady State Ordered Theorell-Chance, or Rapid-Equilibrium Random bi-bi mechanism [23,24]. The intercept replots were always linear (inset, Figures 2 and 3), yielding the kinetic constants shown in Table 2.

DISCUSSION

Few studies have reported GSTs from crustaceans, including the purification and characterization of GST isoforms. *L. vannamei* is one of the shrimp species that has received more attention in the last decade, owing to its intensive production. GSTs from a variety of sources have been purified using conventional chromatographic procedures, as well as by

TABLE 1. Activities and Protein Concentration During *L. vannamei* GST Purification

Purification Step	*Volume (mL)*	*Protein (mg/mL)*	*Total Protein (mg)*	*Specific Activity (μmol/(min mg))*	*Total Activity (μmol/(min mL))*	*Recovery (%)*	*Purification Fold*
Gills extract	10	17.33	173.3	4.15	720.23	100	1
Clarified gills extract	9	4.66	41.94	15.86	665.17	92.36	3.48
Affinity chromatography	1	0.237	0.237	440.12	104.31	14.48	106

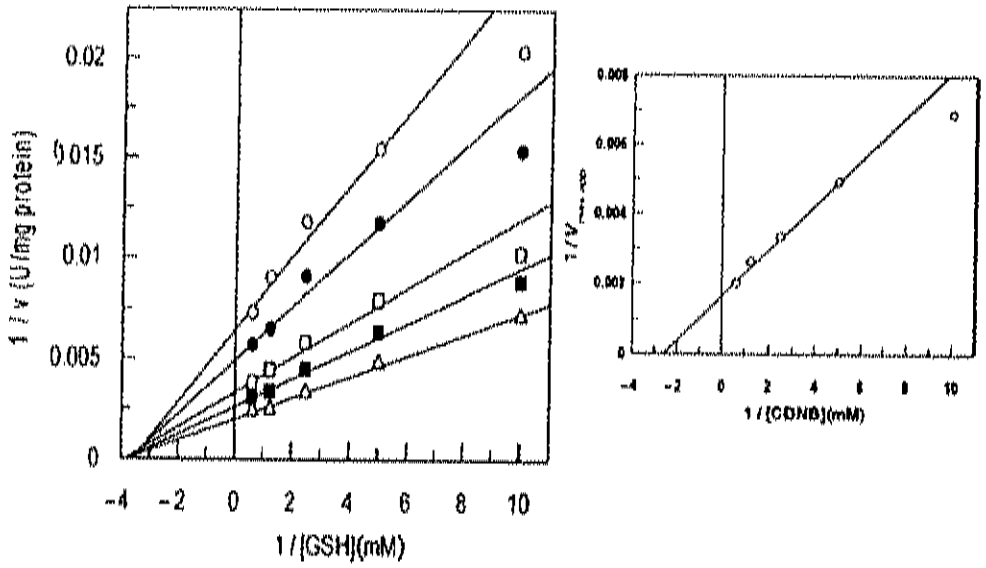

FIGURE 2. Initial velocity pattern for the *L. vannamei* GST. Different GSH concentrations and fixed concentration of CDNB. The concentration of CDNB was 0.1 mM (○), 0.2 mM (●), 0.4 mM (□), 0.8 mM (■), and 1.6 mM (△). Assay mixtures contained 1 μg of enzyme in 1 mL reaction mixture. Rates have been corrected for background reaction. A secondary plot of data from a Lineweaver–Burke plot showing the effect of varying CDNB concentration as ordinate intercept vs. 1/[CDNB].

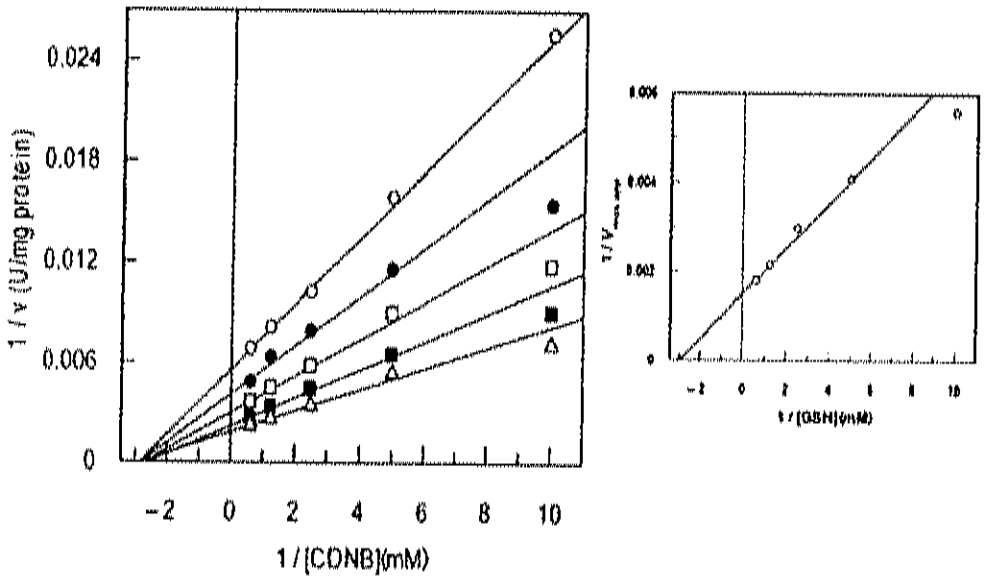

FIGURE 3. Initial velocity pattern for the *L. vannamei* GST. Different concentrations of CDNB and fixed concentration of GSH. The concentration of GSH was 0.1 mM (○), 0.2 mM (●), 0.4 mM (□), 0.8 mM (■), and 1.6 mM (△). Assay mixtures contained 1 μg of enzyme in 1 mL reaction mixture. Rates have been corrected for background reaction. A secondary plot of data from Lineweaver–Burke plot showing the effect of varying GSH concentration as ordinate intercept vs. 1/[GSH].

TABLE 2. Kinetic Constants of Purified GST From Gills of *L. vannamei* with GSH and CDNB

	V_{max} (μmol/(min mg))	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
CDNB	602.90 ± 38.06	0.3904 ± 0.065)	251.2	642.93
GSH	659.38 ± 35.47	0.3350 ± 0.0502	274.4	820.12

Each value represents the mean of four independent determinations ± standard error.
1 μg of purified GST was used *per* assay mixture.

affinity chromatography. Since most cells contain several glutathione-binding proteins, when GSH-affinity chromatography is used, generally more than one purification step is necessary owing to the presence of isoforms and classes of GSTs [25] in addition to other GSH-binding enzymes. Nevertheless, the shrimp GST was successfully purified using GSH affinity. These results agree with data from other nonvertebrate GSTs [16]. It is also known that some GST isoforms do not bind GSH-affinity matrices, in contrast to GSTs classified into Alpha, Mu, and Pi classes [16]. In fact, the first fraction eluted from the GSH-affinity column had a slightly larger protein, although in minute quantity, which might be another GST (data not shown). The spectropholometric detection of the GST activity agrees with the detection in the zymogram. Reduced GSH was used for successful elution from the column, perhaps also aiding as a noninhibitory and stabilization compound for the enzyme [16].

There are few reports about GST purification and kinetic characterization in crustaceans. In the blue crab *Callinectes sapidus*, two different acidic and dimeric GSTs were purified from the F cells of hepatopancreas using gel filtration, ionic exchange, and chromatofocusing [26]. Also, from the hepatopancreas of crayfish *Macrobrachium vollenhovenii*, GST was purified by ion-exchange chromatography and gel filtration and suggested to be similar to the Omega and Zeta classes of GSTs. [27]. The *M. vollenhovenii* GST had a specific activity of 12.36 μmol/(min mg) protein [27], after 43-fold purification, contrasting with the larger values for the shrimp gills GST, which had an activity of 440.12 μmol/(min mg) after 106-fold purification with one chromatographic step using CDNB as a substrate. The values are also high compared to GST1 from another marine invertebrate, the blue mussel *Mytilus edulis*, which had an activity of 10.05 μmol/(min mg) [5]. Moreover, vertebrate Mu GST activities (also toward CDNB) are also lower in the rat GST, 58 μmol/(min mg) [28]; the mouse GST, 68.6–98.4 μmol/(min mg); Atlantic salmon GST, 0.290 μmol/(min mg) [29], and white sturgeon GST, 2.4 μmol/(min mg) [30]. In addition to the ability to conjugate electrophilic substrates to glutathione, GSTs can have a wide array of other functions, such as peroxidase and isomerase activities, and can inhibit the Jun N-terminal kinase [31]. Perhaps, the high specific activity of shrimp GST is related to the presence of other activities, but this needs to be investigated.

Glutathione *S*-transferase kinetic mechanism can be complex. Under low GSH concentrations (<0.1 mM), the initial velocity patterns suggest a ping-pong kinetic mechanism. At high concentrations, an ordered sequential mechanism predominates, where glutathione is the first substrate to react with the enzyme [19]. The intersecting patterns, for the initial velocities of *L. vannamei*, rule out a ping-pong kinetic mechanism and are consistent with the sequential mechanism [23]. The fact that the common intersection point lies on the abscissa indicates that the subunits are catalytically equivalent and that the binding of one substrate does not affect the binding of the other, as reported for a GST from the crayfish *M. vollenhovenii* [27].

Values of K_m for CDNB and GSH are very similar in this study (390 and 335 μM, respectively), which may suggest a random mechanism since there is no significant difference on the substrate affinity. Invertebrates' GST kinetic parameters have been determined by a variety of experimental approaches, but these limit the comparisons between species. The K_m values obtained in nonvertebrate organisms using CDNB as substrate have affinities between 50 and 1800 μM [16], and for GSH these values are from 60 to 2700 μM. This is in agreement with *L. vannamei* K_m values.

The turnover number, k_{cat}, and the catalytic efficiency, k_{cat}/K_m, for *L. vannamei* GST were obtained. Since few studies report these kinetic constants for crustaceans' GSTs, the comparisons to the *L. vannamei* enzyme remained limited. For shrimp, we found values of $k_{cat(CNDB)}$ of 251.2 s^{-1} and $k_{cat(GSH)}$ of 274.4 s^{-1}, while for the clam *R. decussates*, Hoarau et al. [14] found k_{cat} values of 214–250 s^{-1} for seven GST isoforms, and k_{cat}/K_m of 44.4–672.95 and 81.34–1244.97 mM^{-1} s^{-1} for GSH an CDNB respectively. Adewale and Afolayan [27] showed that the turnover of a GST from the crayfish *M. vollenhovenii* was 20.30 s^{-1} for k_{cat}, and 1.60×10^4 M^{-1} s^{-1} for $k_{cat}/K_{m(GSH)}$ and 1.01×10^4 M^{-1} s^{-1} for $k_{cat}/K_{m(CDNB)}$. Overall, the values for these parameters in case of *L. vannamei* are in good agreement with the data previously reported.

Expression of GST isoforms may be tissue-specific in responding to the nature and environmental exposure of the tissues. Since GSTs from the hepatopancreas of marine invertebrates such as *M. vollenhovenii* [27] and *R. decussatus* [14] have lower affinity toward the substrate (electrophile), compared to the GST purified from gills of shrimp, different isoforms may have been purified. Gills are in direct exposure to the contaminants present in the environment; therefore, specific isozymes with higher affinities, as well as induction at transcription levels, may be responsible for the higher detoxification capabilities of marine animals' gills.

In summary, a Mu-class GST was purified from gills of *L. vannamei* using affinity chromatography and its identity was validated with the cDNA deduced amino acid sequence. The enzyme appears to be a homodimer and has high specific activity compared to other GSTs. Even though the enzyme appears to follow a random kinetic mechanism, further experiments, including inhibition studies, are necessary to elucidate

the exact mechanism. It remains to be investigated if this enzyme has other functions such as peroxidase and isomerase activities that have been reported for vertebrate GSTs.

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3

Role of Invariant Tyrosines in a Crustacean Class Mu Glutathione S-Transferase from Shrimp *L. vannamei*: Site-directed Mutagenesis of Y7 and Y116

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Para: Biochemical and Biophysical Acta: Proteins Structure and Proteomics

Role of invariant tyrosines in a crustacean mu-class glutathione S-transferase from shrimp *Litopenaeus vannamei*: site-directed mutagenesis of Y7 and Y116

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Keywords: glutathione transferase, mutants, Y7F, Y116F, shrimp, mu-class GST, *Litopenaeus vannamei*

Abstract

Y6 and Y115 are key amino acids involved in enzyme-substrate interactions required for catalysis in mu-class glutathione S-transferase (GSTs). Those residues provide electrophilic assistance and stabilize substrates though their hydroxyl groups. Two site-directed mutants (Y7F and Y116F) were made to study the role of equivalent residues in the active site of the shrimp *Litopenaeus vannamei* mu-class GST. Both mutants and wild-type enzymes were expressed in *E. coli* and purified by GSH affinity chromatography. The mutants and wild type enzymes catalytic steady-state parameters were determined using CDNB as the second substrate. The sequences were modeled based on a crystal structure of a mu-class GST to provide further insights of the changes at the active site. The Y116F mutant had a small increase in k_{cat} and barely changed its k_{cat}/K_m for GSH and CDNB compared to wild-type, similarly with Y7F. Molecular modeling showed that the shrimp GST theoretical model has a H108 residue that may contribute to compensate and lead to a less deleterious change when conserved tyrosine residues are mutated. This work indicates that shrimp GST is a useful model to understand catalysis mechanisms in this critical enzyme.

1. Introduction

Glutathione transferases (GST; E. C. 2.5.1.18) are a diverse family of enzymes found ubiquitously in aerobic organisms. They play a central role in the detoxification of both endogenous and xenobiotic compounds and are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress [1, 2]. These enzymes catalyze the conjugation of the tripeptide glutathione to the electrophilic center of lipophilic compounds, increasing their solubility and aiding excretion from the cell [3] (Fig 1). This reaction is considered the initial step in the formation of mercapturic acid, a pathway where hydrophobic xenobiotics are inactivated and eliminated from the body. Several GST forms have been identified and characterized, primarily in mammals. These forms can be grouped into at least six distinct classes named alpha, mu, pi, theta, sigma, and zeta on the basis of the N-terminal sequence, the substrate specificity and the immunological properties [2, 4].

The study of non-mammalian GSTs has lead to the description of new GST classes, such as the beta-class found in bacteria, the phi- and tau-classes of plants, as well as the delta-class described in insects [5]. GST activity has also been detected in a large number of invertebrate species, where the tolerance of these organisms to the toxicity of foreign chemicals in the aquatic environment has often been related to their GST content [6, 7]. The relationship between GSTs structure and functional properties is a central issue to understand their participation in xenobiotic metabolism.

There are reports describing the expression and induction of GSTs after exposure to toxic chemicals [8-10], although marine invertebrate GSTs are not as well studied as vertebrate GSTs. Few studies report purification and characterization of this enzyme from marine invertebrates, such as the clam *Ruditapes decussates* [11], the shrimp *Penaeus japonicus*

[12] and recently, the purification and kinetic characterization of a mu-class GST from gills of *Litopenaeus vannamei* [13] .

Mechanistic and structural GSTs characterization has led to the study of the residues involved in the enzyme catalytic mechanism. Site-directed mutagenesis has been extensively used for this purpose, mainly in GSTs from human and rat. The residues Y6 and Y115 (in human and rat mu-class GSTs) are located in the GSH-binding site (G-site) and xenobiotic substrate (H-site) respectively, are essential for catalysis. Y6 is involved in activation and stabilization of GSH thiol group [14, 15] and Y115 has been related with electrophilic assistance and the product release step of the enzymatic reaction [16]. In this study, two tyrosine residues potentially involved in catalysis of *L. vannamei* GST were mutated and their implications on the kinetic parameters of the mutant enzymes were analyzed with CDNB as second substrate.

2. Materials and Methods

2.1. Site-directed mutagenesis

The cDNA coding for a mu-class GST from *L. vannamei* (LvGSTmu) was previously reported [17]. LvGSTmu (GenBank number **AY573381**) was PCR-amplified and cloned in the expression vector pET101/D-TOPO (Invitrogen) using the forward primer 5'-CACCATGCTGCCGGTTCTGGGCTAC-3' and 5'-TCATTTTCCCTCAATGATCG-3' as the reverse primer. Site directed mutagenesis was done using the Stratagene QuikChange Site Directed Mutagenesis kit, using 20 ng of plasmid template. The following primers were used to incorporate the mutations into the cDNA, by replacing the TAC codon for tyrosine with the TTT codon for phenylalanine: **Y7F 5'-**ATGCTGCCGGTTCTGGGCTTTTGGAAAACTCGGG-3'; and

Y116F 5'-GCTTTCTTTACCGTCACCTTTGAACACTACGAACAGAAGG-3', the mutated codon is underlined. The amplification parameters were as follows: an initial DNA denaturation at 95°C for 30 s, followed by 15 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min and extension at 68°C for 6 min. After *Dpn* I digestion, the mutated PCR product was used to transform competent XL1 Blue bacteria. Plasmid DNA extraction and purification was done using the GFX PCR DNA and Gel Band Purification kit (Amersham, Biosciences). DNA sequencing was performed to confirm the mutations. All constructs were sequenced at the Genomic Analysis and Technology Core at the University of Arizona, to verify the mutants. The mutant plasmids (Y7FLvGST and Y116FLvGST) and wild type constructs (WTLvGST) were used to transform BL21 Star (DE3) *E. coli* for recombinant expression.

2.2. Heterologous expression and purification

An overnight culture of BL21-transformed cells was diluted 1:20 in 1 liter of LB broth containing 1 % glucose and ampicillin (100 µg/ml), and grown until the OD_{600} reached 0.6 (T0) at 37°C. Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture at a final concentration of 0.5 mM and the incubation was continued for 6 h at 37°C (T6). Bacteria were then harvested by centrifugation at 5000 X *g* for 5 min at 4°C; the bacterial pellet was then washed with a 0.9 % NaCl solution and stored at -20°C until use. The frozen pellet was thawed on ice before being resuspended in a lysis buffer (1 mM EDTA, 1 mM PMSF, 5 mM benzamidine and 5 mM dithiotreithol (DTT) in 100 mM Tris, pH 8.0) using a 4 ml buffer lysis per gram of pellet. The bacterial pellet was lysed by sonication (4 x 15 sec pulses) on ice-cold water bath and the insoluble cell debris was removed by centrifugation at 15,000 X *g* for 30 min at 4°C. The supernatant was clarified

by two sequential centrifugation steps at 22,000 and 45,000 X *g* at 4°C for 30 min each. The supernatant was extensively dialyzed for 24 h at 4°C, with Buffer A (PBS pH 7.3, containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 5 mM DTT, and 1 mM PMSF), before loading on a GSH agarose affinity column for GST purification, as previously described [13]. The purified enzyme was stored at -20°C in Buffer A with 10 % glycerol. Reducing 10-20 % polyacrylamide gradient SDS-PAGE was done using a Bio-Rad Mini Protean III electrophoresis unit. Gels were stained with Coomassie brilliant blue [18] or silver stain [19]. Protein concentration was measured by the method of Bradford [20].

2.3. Steady-state kinetics

GST activity was determined spectrophotometrically, by recording the increase in absorbance at 340 nm. Measurements were made in a Cary 50 Bio UV-Visible spectrophotometer (Varian) at 25°C using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate according to Habig *et al.* [21]. CDNB was dissolved in ethanol. The concentration of ethanol in the reaction mixture was kept constant at 5 % (v/v). The reaction mixture of 1 ml contained in a final concentration, 100 mM sodium phosphate, pH 6.5 and 1 mM each of GSH and CDNB. Each determination was performed in duplicate and corrected for the corresponding non-enzymatic controls. One enzymatic activity unit is defined as the amount of enzyme that catalyses the formation of 1 µmol of product per min under the conditions described above. Specific activity was expressed as micromol product·min^{-1}·mg^{-1} protein. GST activity was calculated using the extinction coefficient E= 9.6 $mM^{-1}cm^{-1}$ [21].

The kinetic parameters were determined for GSH and CDNB substrates by recording the activity towards a concentration range of 0.05–0.8 mM for GSH and 0.2–2.0 mM for

CDNB, varying the concentrations of one substrate while the other was kept constant. The experimental data were fitted to the Michaelis-Menten equation, by using the nonlinear regression analysis program GraFit v 4.0.21 (Erithacus Software).

2.4. Molecular modeling

Several amino acid sequences of mu-class GSTs, similar to shrimp GST were found in the Protein Data Bank by using the BLAST algorithm [22]. The human mu–class GST (M1-1) isoenzyme was selected as the best template for modeling the shrimp GST (PDB entry **1XWK**).

Molecular modeling was started by submission of the shrimp GST sequence to the Swiss-Model Server [23, 24], using the human GST coordinates as a crystallographic structure template. The model for the mutants was made by changing the structure of residues Y7 and Y116 for phenylalanine using the molecular modeling program O version 8.0.11 [25]. Wild-type and mutant models were structurally optimized with five cycles of idealized refinement with the Refmac5 program [26] of the CCP4 software suite [27, 28]. The final coordinates in the PDB file format were corrected with the MOLEMAN program [29]. The structural model figures were made with the Bobscript program [30, 31]. All the molecular modeling and graphics programs were run either on a Windows NT Silicon Graphics 320 workstation or a Mandrake Linux Pentium III server.

3. Results

3.1 Expression and purification

Y7 and Y116 in LvGSTmu were substituted independently with phenylalanine by site directed mutagenesis to investigate their role in catalysis. The WT and mutant GSTs were

properly expressed in *E. coli* since the induced bacterial crude cells extract had CDNB-conjugating activity in the soluble extract. The expressed wild type and mutant enzymes were isolated and purified by affinity chromatography on immobilized GSH. Induction of the expression constructs resulted in a yield of approximately 67.2 μg and 84 μg of recombinant protein per liter of culture, for Y7F and Y116, respectively. For the WTLvGST recombinant enzyme, the amount purified is 90 μg per liter of culture. Compared to the total activity in the clarified lysate of *E. coli*, the activity recovery for WT was 58%, while for Y7F and Y116F it was approximately 30 %. This agrees with the lower affinity toward GSH of the mutants compared to the WT. The recombinant wild type GST was purified to homogeneity as judged by the appearance of a single band on a reducing SDS-PAGE with an apparent molecular weight of 25 kDa (Fig. 2). Unique bands of the same size were also detected for the two mutants (data not shown).

3.2. Kinetic studies

The catalytic mechanism of CDNB conjugation has been the subject of many studies since this reaction is the most commonly used assay for GST activity (Fig 1). Even though CDNB is not a natural substrate, it is widely used to measure overall activities in cell extracts and to characterize specific GSTs [32]. Steady-state kinetics of the recombinant enzyme and mutants was studied with different concentrations of GSH and CDNB. The reaction followed Michaelis-Menten kinetics and the kinetic parameters were determined using Lineweaver-Burk double reciprocal plots with varying CDNB or GSH concentrations. Table 1 summarizes the kinetic parameters for GSH-CDNB conjugation. The catalytic efficiencies of the mutant enzymes with CDNB were in the same order of

magnitude of the GST wild type. Removal of the hydroxyl group of Y7 and Y116 did not have a striking effect on the catalytic properties of the shrimp enzyme LvGST.

The substitutions Y7F and Y116F affected mostly the apparent affinity (K_m) of the enzyme for GSH, and had marginal effect for CDNB. All the parameters did not change more than two-fold compared to the WT, implying that the shrimp mutants still have significant enzymatic activity. The turnover number resulted with slight differences between wild type GST and mutants, but with a higher values than those reported for the same mutants in hGSTM1 [33]. Perhaps, the effect of substitutions is not as relevant as in hGSTM1; in LvGST, the mutant Y116F has a higher catalysis rate than WT. The catalytic efficiency resulted in a 0.6 fold decrease of k_{cat}/K_m^{GSH} for Y7F and Y116, whereas the k_{cat}/K_m^{CDNB} was similar to that of the wild type or scarcely affected in both mutants.

Molecular modeling was straightforward and models built for both mutants were essentially identical to the theoretical WT shrimp model (Figure 3, panels b and c).

4. Discussion

Site-directed mutagenesis studies have revealed the importance of specific residues in GSTs catalyzed reactions. These studies provide a structural basis for the enzymatic activity and to propose a catalytic mechanism. The catalysis by GSTs usually involves the activation of an enzyme-bound GSH to stabilize a GSH-thiolate anion for reaction with the second substrate [34]. Increasing the reactivity of a thiol can be accomplished by promoting its ionization (lowering its pK_a) and by the desolvation of the resulting thiolate anion. A tyrosine residue (Y6 of rat mu-class GST) in the GSH binding site has been proposed to activate the GSH thiol group for the nucleophilic attack [35, 36].

We used molecular modeling as an approach to obtain structural insights of the active site and the effect of the mutation on catalysis. Although it cannot substitute the crystallographic structural determination, molecular modeling helped to understand the unexpected marginal changes in kinetic parameters. In shrimp GST, residue Y7 is conserved as the main interaction between GSH substrate and protein (Fig. 3a). Within the limitations that a theoretical model has, the hydroxyl group of Y7 was localized at one hydrogen bond distance of the GSH thiol group, suggesting a role in its stabilization as previously reported for mammalian mu-class GSTs [16, 37]. Removal of the Y6-hydroxyl group of rat GST had an important effect on the catalytic properties of the enzyme [34]. However, the corresponding Y7F mutant in shrimp LvGST results only in 40 % of the WT catalytic efficiency. In the molecular model, another tyrosine residue was found localized in the catalytic pocket of LvGST, spatially close to the Y7 residue and the thiol group of GSH (Fig. 3b). This Y210 residue is situated in the variable C-terminal domain, and is not present in other mu-class GSTs. Hence, we hypothesize that Y210 residue could compensate the loss of the hydroxyl group in the Y7F mutant during GSH stabilization, explaining why this mutation is not as deleterious as in mammalian mu-class GSTs.

No positively charged residues are close enough to the GSH sulfur atom to help stabilize a thiolate anion, as in rat GST M3-3 [34]. Besides the Y7 residue in the active site, a histidine residue is present in the shrimp mu-class GST (H108). It is rather interesting to note the presence of this residue, because it is also present in hGSTM1-1 (His107) (Fig. 4), although most mamalian GSTs have an arginine at that position [38]. Recent studies show that this residue is involved in the stabilization of the Meisenheimer complex via interactions with the *ortho*-nitro group of CDNB, and probably functions as the proton acceptor/electron donor in the first step of catalysis [33]. This could suggest a similar role in the LvGST

catalysis, because it is localized approximately at the same distance of CDNB as in hGSTM1-1.

Desolvation of the active site increases the reactivity of the GSH thiol group. For the rat GST, this occurs by other hydrogen-bonding interactions with the sulfur, specifically with one or two water molecules in the solvation shell [36]. Additionally, the sulfur is closer to the side chain of L12 and to the edge of the aromatic ring of Y6. These two van der Waals interactions shield a large portion of the sulfur atom from solvent [39]. In WTLvGST, these van der Waals interactions between L13 and Y7 are conserved and may contribute to substrate stability. Also, the L13 residue appears to be relevant for the structural integrity of the H and G sites, as described in hGSTM1 [33]. Further investigation on this residue of LvGST could confirm this.

The hydroxyl group of Y115 (in other mu-class GSTs) is also involved in the product release step of the enzymatic reaction. When the product release is the rate-limiting step in the turnover, as it is with CDNB, the Y115F mutant has a higher turnover than the native enzyme [16]. It is most likely possible that the hydrogen-bonding interactions between the hydroxyl groups of Y115 (as hydrogen bond donor) and S209 (in rat mu-class GST), which help keep together the α4 helix and the C-terminal tail of the protein, decrease the segmental motion of the protein and inhibit product release as well [40].

In LvGST, the turnover number results with slight differences between the wild type GST and the mutants, but their values are higher than those reported for the same mutants in hGSTM1 [33]. Perhaps, the effect of substitutions is not as marked as in hGSTM1; in LvGST mutant Y116F (Fig. 3c) results with a higher catalysis rate than in wild type. This behavior is consistent with that reported for the same mutants in other mu-class GSTs [16,

33]. However, the catalytic efficiency resulted slightly decreased for Y116 of k_{cat}/K_m^{GSH}, and the k_{cat}/K_m^{CDNB} was similar to that of the wild type.

Also, the S209 residue is not present in LvGST; instead, the C207 residue is present with a lack of any interaction with Y116, because it is located farther than a hydrogen bond distance. This may be an indicative of a higher release of products out of the enzyme, compared to other GSTs (k_{cat} higher in LvGST), due to the interaction elimination between residues that keep together the 4α helix and the C-terminal tail. Consequently, comparing the mutants with the wild type, there are no relevant differences neither in the catalysis rate nor in the catalytic efficiency, because of the lack of the interaction with a residue like the S209 residue in either the presence or absence of the hydroxyl group. In addition, the F112 residue appears to maintain the size of the H-site.

Y115 has been identified as a residue that provides electrophilic assistance by its hydroxyl group in the addition of GSH to xenobiotic substrate. A higher distance is predicted from the model for the GST of the *L. vannamei*, between the H-site Y116 residue and the xenobiotic, moving even farther than a hydrogen bond away from the substrate CDNB and eliminating any possibility for a direct interaction (Fig. 3). Thus, same as for the Y7 residue, the OH group elimination from the Y116F mutant does not markedly affect the catalysis constant values, which may indicate a lack of electrophilic assistance from this residue during the reaction.

Finally, no drastic changes were observed in neither of both mutants regarding the native enzyme. Despite the catalysis rate resulted a little higher for the Y116F mutant, a slightly higher catalytic efficiency was indeed observed for the WT, because of its higher affinity for both substrates. This indicates that the role represented by these residues in the shrimp GST may not be as relevant (by itself) for the catalysis of this enzyme. For the catalytic

performance of the enzyme, the participation of other residues from the active site becomes necessary, where the H108 could certainly perform an important role, since it has been proposed to work as a general base by lowering the pK_a value of the GSH enzyme bound. Site-directed mutagenesis studies towards other active site residues would provide more information concerning the shrimp GST catalytic mechanism, such as the F112 and the Y210. Both of these residues are found in the binding site to the xenobiotic substrate and could have some kind of interaction with the substrate, since they are closer to it than the Y116. It could be implied that the Y210 may be performing the function of providing electrophilic assistance to the substrate instead of being the Y116, or may replace its function when the later is not present. Other residues that also appear to be critical for catalysis may be subject for mutagenesis and structural studies, and will set this enzyme as an alternative system in order to understand the catalysis and the enzyme function under the marine invertebrates evolutive perspective.

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Figure Legends

Fig 1. Catalytic mechanism of glutathione-S-transferase. (Taken form Johnson et al., 1993 [16])

Fig.2. SDS-PAGE of recombinant wild type GST purification. Lane 1, MW markers (BioRad); lane 2, clarified cell lysate; lane 3, purified recombinant GST.

Fig. 3. Stereoview of the theoretical model of shrimp LvGSTmu active site with GS-DNB conjugate. a) Wild type (WT). b) Y7F mutant. c) Y116F mutant. GS-DNB is shown in filled bonds.

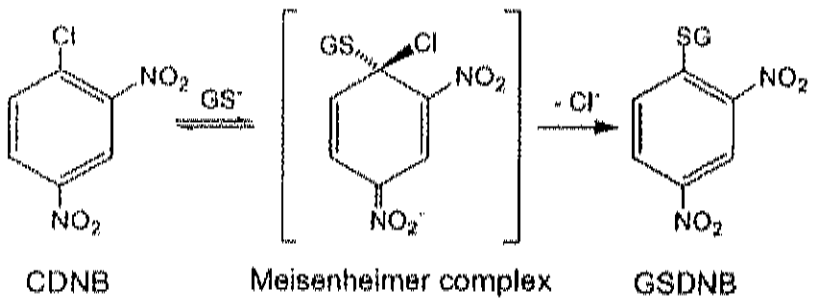
Fig. 4. Stereoview of the superimposition of active sites residues of LvGSTmu (thick lines) and hGSTM1-1 (thin lines), both complexed with GS-DNB. Active site residues of hGSTM1-1 were taken from the coordinates of pdb **1WXK**. Note the different orientation of the CDNB ring.

Tables

Table 1. Kinetic parameters of LvGSTmu mutants with CDNB as second substrate

Mutation	substrate	V_{max} ($\mu mol/min^{-1}\ mg^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
WT	GSH	484.7 ± 14.8	0.068 ± 0.008	201.9	2931
	CDNB	419.3 ± 47.4	2.29 ± 0.42	174.7	76.3
Y7F	GSH	303.9 ± 20.4	0.105 ± 0.023	126.6	1206
	CDNB	302.8 ± 52.8	2.26 ± 0.65	126.2	55.6
Y116F	GSH	685.3 ± 30.2	0.247 ± 0.026	285.5	1155
	CDNB	644.9 ± 98.0	3.24 ± 0.72	268.7	82.9

Figure 1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NO2
GS-
GS
Cl
NO2
NO2
- Cl-
SG
NO2
NO2
CDNB
Meisenheimer complex
GSDNB

Figure 2

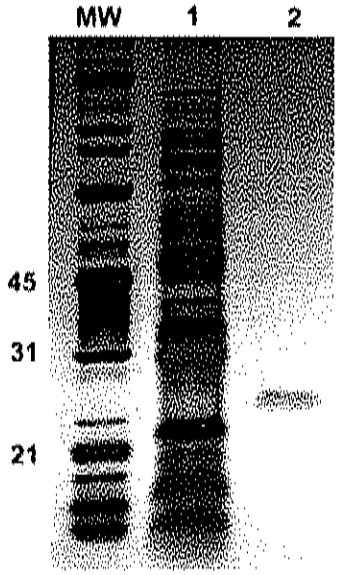
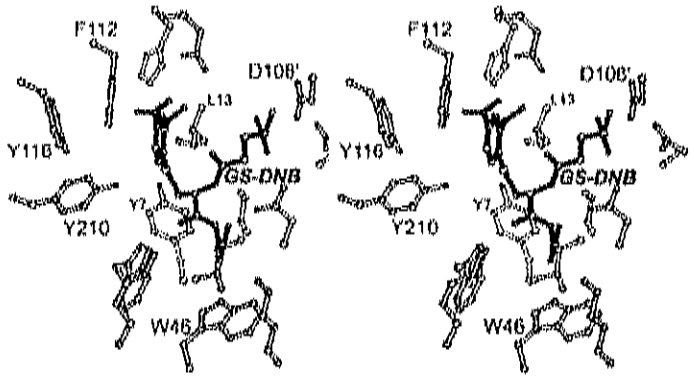
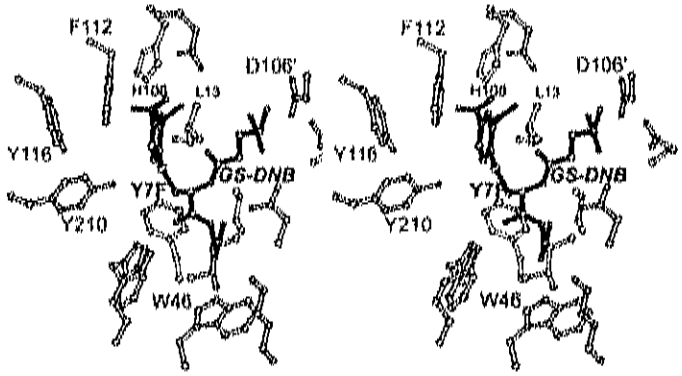


Figure 3

a)


F112
D106'
L13
Y116
GS-DNB
Y7
Y210
W46
F112
D106'
L13
Y116
GS-DNB
Y7
Y210
W46

b)


F112
D106'
H106
L13
Y116
GS-DNB
Y7F
Y210
W46
F112
D106'
H106
L13
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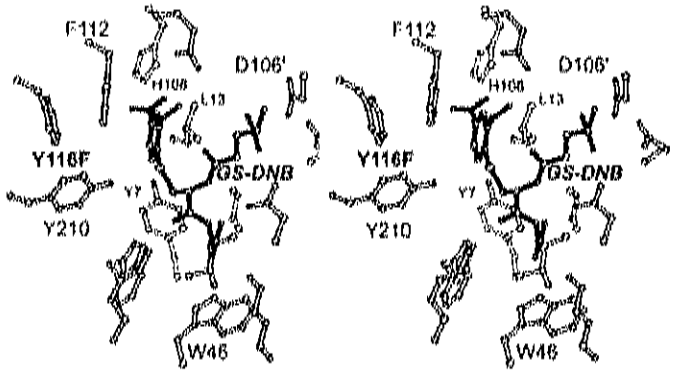
Y210
W46

c)


F112
D106'
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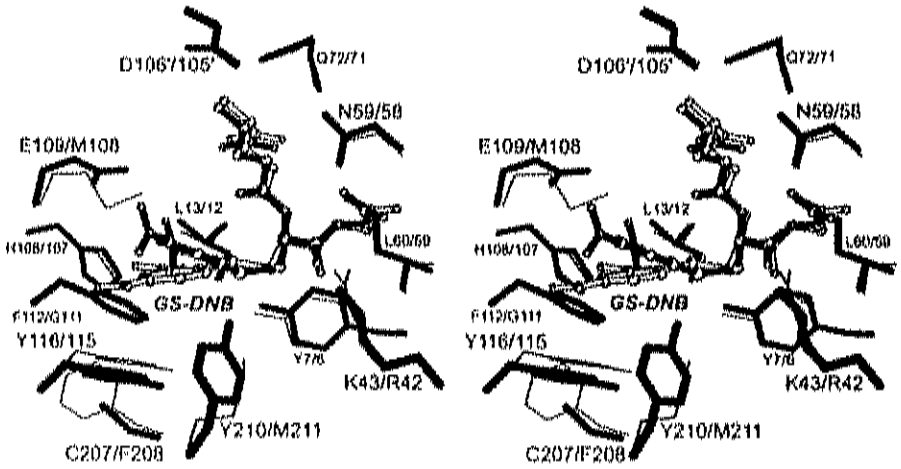
L13
Y116F
GS-DNB
Y7
Y210
W46

Figure 4


D106'/105'
Q72/71
N59/58
E109/M108
L13/12
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L60/59
F112/G111
GS-DNB
Y116/115
Y7/6
K43/R42
Y210/M211
C207/F208
D106'/105'
Q72/71
N59/58
E109/M108
L13/12
H108/107
L60/59
F112/G111
GS-DNB
Y116/115
Y7/6
K43/R42
Y210/M211
C207/F208

Anexo

A Mu-Class Glutathione *S*-Transferase from the Marine Shrimp *Litopenaeus vannamei*: Molecular Cloning and Active-Site Structural Modeling

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ABSTRACT: A cDNA clone coding for a mu-class glutathione *S*-transferase (GST) was isolated from a hepatopancreas cDNA library from the shrimp *Litopenaeus vannamei*. The deduced amino acid sequence (215 amino acids) has >50% identity to rodents and other mammals mu-class GSTs. Using RT-PCR, the shrimp GST transcript was detected in hepatopancreas, hemocytes, gills, and muscle, but not in pleopods. The shrimp GST sequence was computer modeled and found to fit the classical two-domain GST structure. Domain I, containing the glutathione (GSH) binding site, is more conserved compared to the flexible C-terminal domain II. Residue Q208 appears to be a key to substrate specificity by comparison with mammalian GST mutants. This position is commonly occupied by serine or threonine in mammalian mu-class GSTs, and shrimp Q208 may affect the affinity to substrates like aminochrome or 1,3-dimethyl-2-cyano-1-nitrosoguanidine. This is the first report of molecular cloning and structural modeling of a crustacean GST and provides new insights into the nature of the detoxification response on marine invertebrates. © 2004 Wiley Periodicals, Inc. J Biochem Mol Toxicol 18:245–252, 2004; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20033

KEYWORDS: cDNA; Glutathione *S*-Transferase; Shrimp; Prawn; Mu-class GST; *Penaeus, Litopenaeus vannamei*; Molecular Modeling

INTRODUCTION

Glutathione *S*-transferases (GST E.C. 2.5.1.18) are a family of enzymes that utilize glutathione (GSH) as a substrate in reactions that permit the biotransformation and disposal of a wide range of exogenous and endogenous compounds [1]. These compounds may be xenobiotics, drugs, or products of oxidative stress. GSTs catalyze the conjugation of electrophiles to the reactive thiol of glutathione, converting the electrophile into a more water-soluble product, which can be metabolized into a mercapturic acid for urinary excretion [2]. These proteins have been found in virtually all organisms studied, including plants, animals, and bacteria. Eukaryotes usually contain multiple GSTs with different catalytic abilities to accommodate a wide range of functions within the cells [3]. Mammalian cytosolic GSTs are grouped into different classes primarily based on protein sequence similarity and substrate specificity and are named as alpha, mu, pi, theta, zeta, sigma, and omega [2,3]. Additionally, the kappa GST is a soluble mitochondrial enzyme, and new classes of GSTs have been found in nonmammalian species, such as the beta-class found in bacteria, the phi- and tau-classes in plants, and the delta-class described in insects [4].

Cytosolic GSTs are dimers, with subunits of approximately 25 kDa [3], each one containing a GSH-binding site (the G-site) and a hydrophobic substrate-binding site (the H-site). While the G-site is well conserved among GSTs, the H-site varies widely in different classes, leading to differences in substrate

The sequence reported in this paper has been submitted to GenBank and is available under accession number AY573381.

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selectivity [2]. In humans and rodents, GSTs are expressed in several tissues, and are particularly abundant in liver, where they can constitute as much as 4% of total soluble proteins [3].

Invertebrate GSTs appear to be also expressed in several tissues and different developmental stages, although they have not been studied as much as the vertebrate counterparts [5]. Resistance of several invertebrate species to toxic compounds such as pesticides and environmental contaminants appears to be mediated, at least in part, by GSTs. DDT resistance in the mosquito *Anopheles gambiae* is mediated in part by a novel GST gene named *aggst3-2* [6]. Similarly, the gene *nlgst-1* coding for an insect class 1 GST is overexpressed in the rice brown plant hopper *Nilaparvata lugeons* pyrethroid-resistant strain [7]. Compared to mammals and insect, marine invertebrate GSTs are less known, although reports about their induction after exposure to toxic chemicals are becoming available. Induced GST activity has been reported in the water flea *Daphnia magna* after exposure to phenobarbital [8], in the crab *Carcinus maenas* after treatment with cypermethrin [9], and in the abalone *Haliotis rufescens* and black chiton *Katharina tunicate*, GST activity increased upon treatment with brominated phenol [10]. Recently, in a clam *Ruditapes decussatus* several GST isoforms were described; interestingly, the N-terminus of one of the subunits has a sequence similar to alpha/mu/pi GST from *Fasciola hepatica* [11]. In the shrimp *Penaeus japonicus*, two anionic GSTs were purified from eye tissue and one of the N-terminus sequences was similar to mu and the other to theta-class GST [12]. However, to our knowledge, nothing else is known about their sequence, expression, and structure.

Structural information about mu-class GSTs is available for several mammalian species [13–17]. Since three-dimensional structure determination of proteins is a complex and time-consuming process [18], molecular modeling is a very good alternative to experimental structure assessment. The only requirement is that the target amino acid sequence is 40% or more identical to a known template protein structure [19]. Homology modeling has provided key insights into GSTs [20]. Comparative studies have shown that the three-dimensional structure of GSTs from different species and classes are remarkably similar to the glutathione binding site or domain I [14,15,21–23]. However, the more variable domain II plays an important role in the substrate specificity [13,24,25]. Therefore, the molecular modeling complements and provides key hypothesis and insights into the future work once the amino acid sequence has been determined. In this report, we present the molecular cloning, expression, and a molecular model of a mu-class GST from the shrimp *Litopenaeus vannamei*.

MATERIALS AND METHODS

Cloning, Sequencing, and Sequence analysis

A hepatopancreas cDNA library from *L. vannamei* was constructed [26] using the unidirectional Zap Express synthesis system (Stratagene). Isolated clones were obtained by in vivo excision using the ExAssist helper phage and the *Escherichia coli* XLOLR strains according to the manufacturer instructions (Stratagene). Plasmid DNA was purified using the alkaline method [27] and was used for sequencing with the T3 and T7 primers that flank the multiple cloning sites and with internal primers GST-FW and GST-Rv, in an ABI 337 automated DNA sequencer at the Laboratory of Molecular and Systematic Evolution Facility at the University of Arizona (Tucson, AZ). Internal primers GST-Fw (5′-CCAGACTACGACAAGAGCGAATG-3′) and GST-Rv (5′-TAGGCCAGGAAGTCGATGTAGG-3′) corresponding to positions 137–159 and 498 to 476 (complementary strand), respectively, were designed based on the sequence obtained from the clone and used also to thoroughly sequence both strands (100%). Identity of the clone was assigned by comparison with the unambiguous sequence against GenBank using the BLASTX algorithm [28]. Multiple sequence alignments of the GST sequence to other organisms were done with CLUSTAL W [29], and the figures were made using BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

RNA Isolation and RT-PCR

Total RNA was isolated from *P. vannamei* tissues (hepatopancreas, hemocytes, muscle, pleopods, and gills) using the TRIZOL reagent according to manufacturer instructions (Invitrogen). Five μg of the total RNA was subjected to reverse transcription (RT) at 42°C in the presence of oligo-dT and SuperScript II reverse transcriptase (Invitrogen,) in a 20 μL reaction volume, according to the instructions of the manufacturer. PCR amplifications were done using 2.5 μL of the RT reaction, 1 U of platinum *Taq* polymerase (Invitrogen), 20 pmol of each forward and reverse primers, 1.5 mM $MgCl_2$, and 200 μM of each dNTP in PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3). Amplifications were done as follows: 1 min, 94°C; 1 min, 55°C; and 3 min at 72°C, for one cycle and then for 34 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C, and a final extension for 10 min at 72°C. RT-PCR for a ribosomal protein L21 mRNA (GenBank accession no. BE188654), using the primers L21-Fw (5′-GGGCTTCTTTCCCGTTTCAGC-3′) and L21-Rv (5′-GCAATGAACTCATAAGGCAGTGGC-3′) as

previously described [30] was included for comparisons. The amplified PCR products were analyzed in a 1.5% agarose gel, stained with ethidium bromide and visualized by UV light.

Molecular Modeling

Several mu-class GST amino acid sequences similar to shrimp GST were found in GenBank using the BLAST algorithm [28] and the PDB subset. A sequence alignment made with Clustal W [29] was used to determine the best template protein on the basis of amino acid sequence similarity. The isoenzyme 3-3 rat GST mu-class was selected as the most similar protein to shrimp GST (PDB entry 6GSU).

Molecular modeling was started by submission of shrimp GST sequence to the Swiss-model server [31–33], using the rat GST coordinates as a crystallographic structure template. The model produced by SwissModel required insertions and deletions to match the shrimp GST sequence. These changes were done with the SwissPdViewer program version 3.51 [34] and the O program version 8.0.11 [35]. O was also used to fix bond distances, angles, and dihedrals. A three-cycle structural refinement was done with the Refmac5 program [36] in the CCP4 graphical interface suite [37,38]. Stereochemistry was verified using the program Procheck 3.4 [39], and the geometrical distortions were fixed using the refinement routines included in the O program. The GST dimer was generated by a two-fold symmetry operation using the SwissPdbViewer program. The ligand modeled in the active site was the reaction product (9R,10R)-9-(*S*-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene (GST-X) as done in the crystal of rat GST [16]. The final coordinates in the PDB file format were corrected with the MolMan program [40,41]. The figure of the structural model was made with Molscript [42] and Raster3D programs [43]. All the molecular modeling and graphics programs were run either on a Windows NT Silicon Graphics 320 workstation or a Mandrake Linux Pentium III server.

RESULTS AND DISCUSSION

Sequence Analysis of the GST cDNA

A full-length cDNA clone, containing the coding region of a mu-class GST, was isolated from a *L. vannamei* hepatopancreas cDNA library, and both strands (100%) were thoroughly sequenced and deposited under GenBank accession no. AY573381. The cDNA clone (847 bp) includes an open reading frame (ORF) of 648 bp flanked by 21-bp 5'UTR and 178-bp 3'UTR, containing a poly A tail of 20 bp (Figure 1). Although the sequence contains the poly A^+ tail, no clear polyadenylation signal was found; however, the sequence AAGAAA occurs in position 748 and differs by one base (T to G) from the classical signal. The deduced protein sequence corresponds to a 215 amino acid protein with predicted molecular weight of 25.18 kDa and pI 8.391. The identity of the predicted protein was suggested by the high similarity with mu-class GSTs, as described below.

The cytosolic GST active site comprises residues of both monomers. The G-site binds GSH with high specificity and is located in domain I close to the N-terminus. In domain II, the H-site binds the hydrophobic substrate and determines the specificity of the enzyme [3,20]. As in other GSTs, the *L. vannamei* GST contains the conserved G-site motif FPNLPYYIDGD between residues 58 and 67 [44]. A multiple alignment of *L. vannamei* GST is shown in Figure 2. The sequence comparison showed ~55% identity with mu class-GST from the mouse *Mus musculus* (P15626) [45], the human *Homo sapiens* (P46439) [46], and the guinea pig *Cavia porcellus* (P16413) [47]; 53% with the hamster *Cricetulus longicaudatus* (Q00285) [48]; 52% with the hen *Gallus gallus* (P20136) [49], and 46% with class alpha GST of the parasite *Fasciola hepatica* (P31670) [50]. All these values are within 50% identity, except for the alpha class GST of *F. hepatica*, which are common among same class GSTs [20,51]. Interestingly, this shrimp GST is more similar to vertebrate GSTs than to insects.

Expression of Shrimp GST by RT-PCR

RT-PCR from several tissues was used to determine the presence of the shrimp GST mRNA in different tissues. The expected DNA product of 362 bp was amplified from hepatopancreas, hemocytes, muscle, and gills RNA, with exception of pleopods (Figure 3). As a control and to roughly estimate the GST mRNA levels among tissues, we compared GST mRNA levels to ribosomal L21, a house-keeping gene, as previously reported [30]. GST is lower than L21 in all the cases, and the transcript is more abundant in hepatopancreas and gills compared to hemocytes and muscle. Hepatopancreas and gills are tissues in close contact with food and water with digestion and oxygen uptake functions, respectively. GST expression patterns correlate well with expression of detoxifying enzymes by tissues with analogous functions to vertebrate liver [5].

Molecular Modeling of Shrimp Mu-Class GST

The amino acids sequence alignment of *L. vannamei* GST and rat mu-class GST has 56% identity. A close-up of shrimp GST active site model is shown in Figure 4. The Ramachandran plot of shrimp GST model indicates

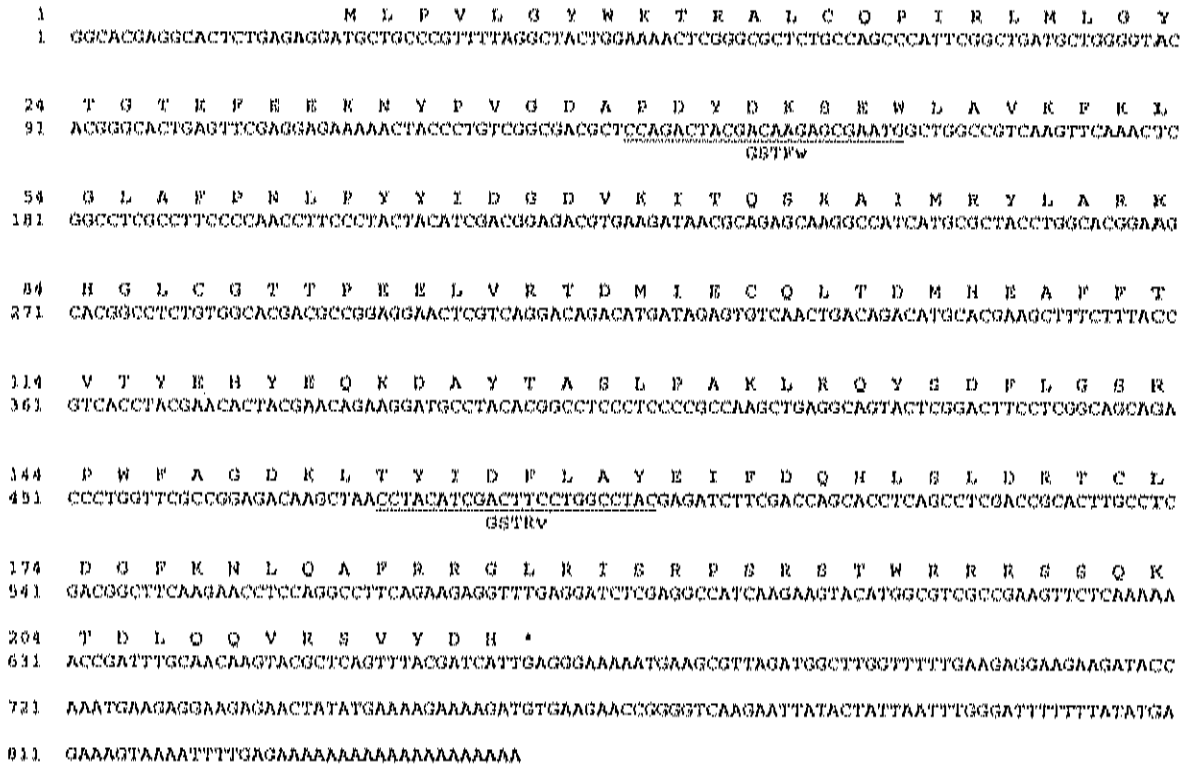

FIGURE 1. Nucleotide and deduced amino acid sequence of the white shrimp GST. Primers used in this work are indicated on the sense strand and underlined. Antisense primer GSTRv is shown in the coding strand. Stop codon is labeled with asterisk.

that 90% of the residues have the most favored dihedrals, and parameters such as bond lengths, angles, and dihedrals are well refined and under limits (data not shown). These values ensure the quality of the model and allow further structural analysis.

The final refined shrimp mu-class GST structural model conserves the general topology folding reported for other GST structures. Domain I (residues 1–74, which comprises four central beta-sheets flanked on one side by two alpha-helices, and a bent, irregular helix structure) is the most similar to the template protein (rat GST PDB 6CSU), since the major amino acids that are reported as decisive in the GSH-binding activity and those that participate in the structure maintenance are conserved in their position and side chain orientation [22]. Meanwhile, in domain II (residues 81–215, globular, and formed by five alpha-helices) there are important differences in the amino acids composition and arrangement compared with the template.

Small differences in secondary structure content between the shrimp and the rat GSTs were found; however, general features such as the positions of a proline between helices 5 and 7 in shrimp GST are also present in the rat GST [22]. Superimposition of alpha-carbon backbones (6CSU vs shrimp GST) gives a root-mean-square deviation (RMSD) of 0.44 Å for domain I and 0.53 Å for domain II. This is consistent with previous comparison studies of alpha/mu/pi GSTs, where more structural divergence is found in domain II [21]. The predicted interactions between domains I and II are mainly between helices 1 and 6, and helices 3 and 4, adding up to 11 contacts (salt bridges or hydrogen bonds) in total. The domains' interaction in rat and avian GST occurs by the same structural elements, but the number of contacts is larger, between 16 and 18 salt or hydrogen bonds [14,22].

Shrimp GST is most likely a dimer where the subunits interact by contacts between domain I of one subunit and the domain II of the other, forming a channel between the monomers. This interface is mostly made of polar side chains and is structurally similar to rat GST. The glutathione binding site (G-Site) has been extensively studied, and in this work we modeled the covalent complex GSH-xenobiotic (9R,10R)-9-(*S*-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene. There is a hydrogen bond between Tyr 7 and the GSH sulfur, as many other contacts with the ligands previously described in crystallographic structures of the complex. The only interaction between GSH and the opposite monomer is a contact between Asp 106 in one subunit and GSH and Gln 72 in the other (Figure 4). The xenobiotic binding

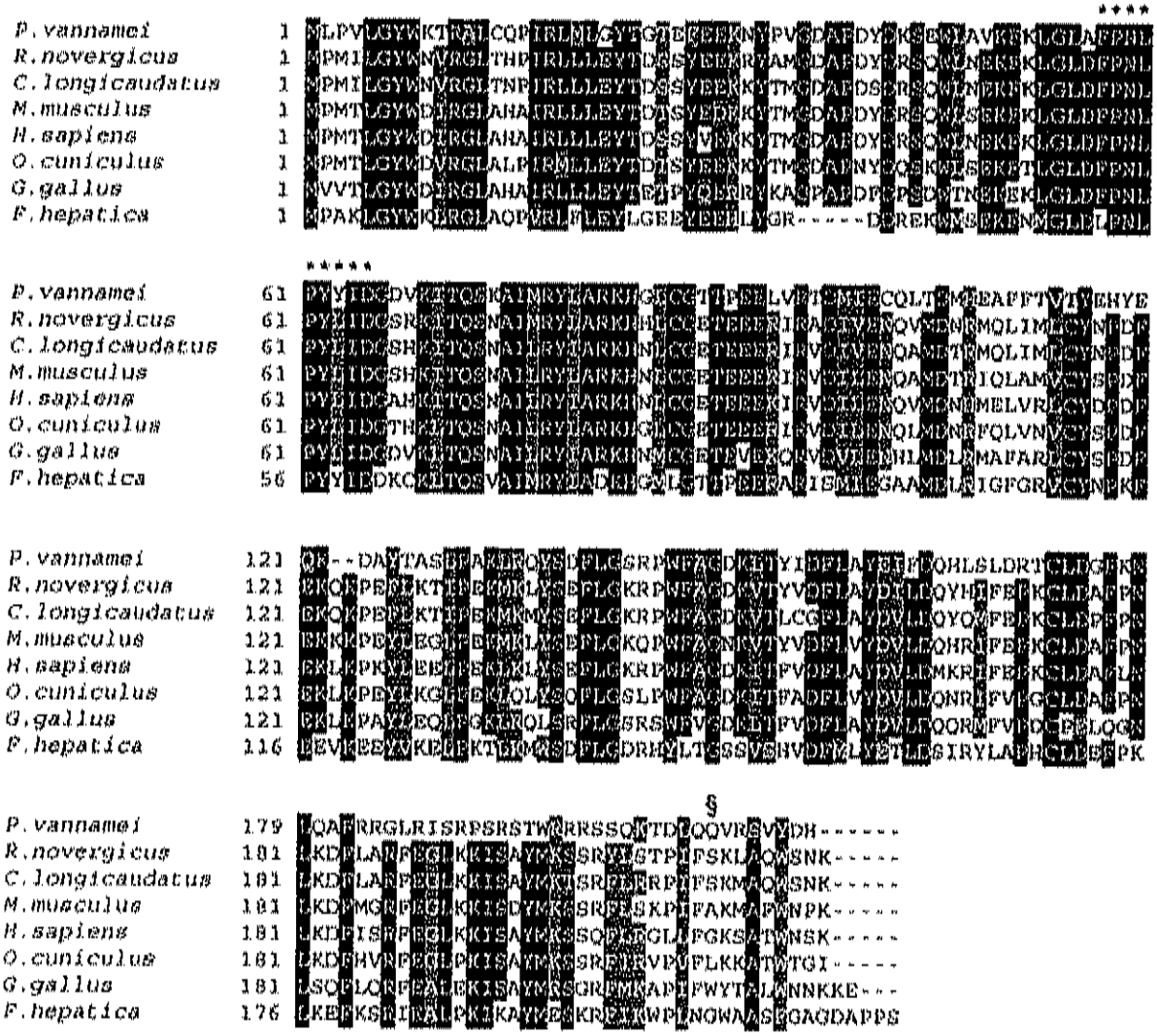

FIGURE 2. Alignment of selected GST amino acid sequences. The C-site region is labeled with asterisks. Position of residue equivalent to position 210 in mammalian GSTs is labeled with §. Shrimp (*L. vannamei*), rat (*Rattus novergicus*, P04905), hamster (*Cricetulus longicaudatus*, Q00285), mouse (*Mus musculus*, P15626), human (P46439), rabbit (*Oryctolagus cuniculus*, P46439), hen (*Gallus gallus*, P20136), and liver parasite (*Fasciola hepatica*, P31670). Identical amino acids are indicated in black regions, and gray regions, are conservative replacements.

site (H-site) is located in domain II, and in mammalian GSTs the contacts between the enzyme and the ligands are mainly Van der Waals interactions. In shrimp GST we found some of these hydrophobic contacts between side chains and the xenobiotic. From Val 10, Ile 111, and Ser 209 in rat GST, which are important for enzyme efficiency and stereoselectivity within mu-class GSTs [14,52], only one site is hydrophobic (shrimp Phe 111). Shrimp Thr 10 and Gln 208 compared to rat Ile 111 and Ser 209 may lead to changes that are reflected in variations of K_m and k_{cat} to specific mu-class GST substrates.

In shrimp GST, there is a larger distance between Tyr 116 and the xenobiotic phenantherene ring (2 Å more compared with rat GST) that may be compensated by the side chain of Gln 208 whose position may maintain the size of the H-site but may increase the polarity within it. However, this distance requires to be confirmed by a direct structural determination since this residue is known to provide electrophilic assistance in addition of GSH to xenobiotic. In addition, we propose that Thr 115 is hydrogen bonded to Ser 209 to maintain bound helix 4 and the C-tail, to decrease its motion, and inhibit the substrate release [13]. In shrimp GST, the C-terminal flexibility should be compensated by the hydrogen bond made by Asp 106. It is known that the C-terminal is flexible and disordered in the presence or absence of GSH, but becomes ordered upon binding to xenobiotics. This structural shift is thought to provide

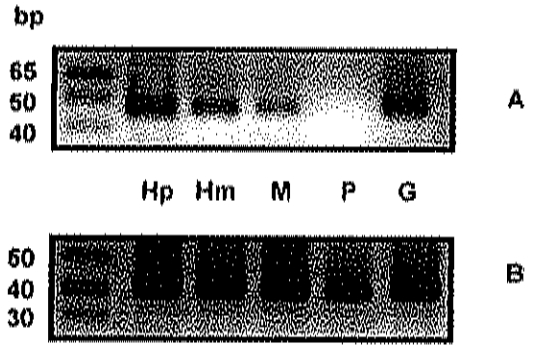

FIGURE 3. Detection of GST transcripts by RT-PCR. Ethidium bromide stained 1.5% agarose gel. *Panel A*: PCR products from hepatopancreas (Hp), hemocytes (Hm), muscle (M), pleopods (P), and gills (G), using GST-Fw and GST-Rv primers. *Panel B*: PCR product of the constitutive ribosomal protein L21 from the same tissues.

a desolvation effect during the catalysis, and involves residue Arg 43 [25], which is conserved in shrimp as Lys 43. We would like to emphasize that the molecular modeling provides many testable hypothesis about reactivity and selectivity of the shrimp GST, and we are in the process of working out these experiments.

Our results open several hypotheses related to the recent work of Ivarsson et al. about mu-class GST

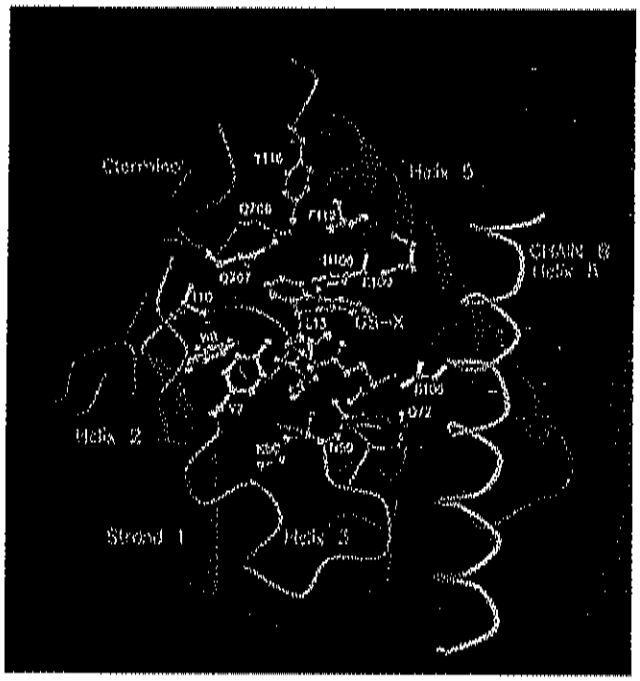

FIGURE 4. Molecular modeling of shrimp GST. Representation of the enzyme active site, residues contacting the ligand, and the position of the alpha-helix from contacting monomer are indicated. GST-X represents the reaction product (9R,10R)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene.

mutants [2]. After a thorough sequence analysis and expression of mutants, several residues responsible for the functional changes between subclasses of this family were identified. Several key residues that enhance the k_{cat}/K_m by several orders of magnitude were found such as the mutant T210S, which in shrimp mu-class GST corresponds to a Gln 208 (Figure 2, position labeled with symbol §). If a conservative mutation such as a threonine to serine leads to a three-orders of magnitude increase in catalytic efficiency to the larger substrate 1-chloro-2,4-dinitrobenzene (CDNB), compared to *trans*-stilbene oxide (tSO), it will be of great interest to determine whether shrimp GST is more capable of using tSO or substrates that may have favorable interactions with the amide side chain of glutamine such as aminochrome or 1,3-dimethyl-2-cyano-1-nitrosoguanidine.

In summary, a GST was found in a marine shrimp and its primary, secondary, and tertiary structures resemble mu-class GSTs. The transcript was detected mainly in hepatopancreas and gills, but albeit lower, also in muscle and hemocytes. The raise of shrimp farming is exposing marine shrimps to artificial as well as natural stress conditions. Frequently, shrimps farms are located many times near agricultural areas where herbicides and pesticides are used; therefore, coping with the stress conditions requires a full detoxifying system where the GST enzymes very likely play an essential role. Whether this shrimp GST or other detoxifying enzymes are induced as well as the mechanisms for induction will await further studies about structure, function, and expression regulation.

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   1  M  L  P  V  L  G  Y  W  K  T  R  A  L  C  Q  P  I  R  L  M
   1  ATGCTGCCCGTTTTAGGCTACTGGAAAACTCGGGCGCTCTGCCAGCCCATTCGGCTGATG

  21  L  G  Y  T  G  T  E  F  E  E  K  N  Y  P  V  G  D  A  P  D
  61  CTGGGGTACACGGGCACTGAGTTCGAGGAGAAAAACTACCCTGTCGGCGACGCTCCAGAC

  41  Y  D  K  S  E  W  L  A  V  K  F  K  L  G  L  A  F  P  N  L
 121  TACGACAAGAGCGAATGGCTGGCCGTCAAGTTCAAACTCGGCCTCGCCTTCCCCAACCTT

  61  P  Y  Y  I  D  G  D  V  K  I  T  Q  S  K  A  I  M  R  Y  L
 181  CCCTACTACATCGACGGAGACGTGAAGATAACGCAGAGCAAGGCCATCATGCGCTACCTG

  81  A  R  K  H  G  L  C  G  T  T  P  E  E  L  V  R  T  D  M  I
 241  GCACGGAAGCACGGCCTCTGTGGCACGACGCCGGAGGAACTCGTCAGGACAGACATGATA

 101  E  C  Q  L  T  D  M  H  E  A  F  F  T  V  T  Y  E  H  Y  E
 301  GAGTGTCAACTGACAGACATGCACGAAGCTTTCTTTACCGTCACCTACGAACACTACGAA

 121  Q  K  D  A  Y  T  A  S  L  P  A  K  L  R  Q  Y  S  D  F  L
 361  CAGAAGGATGCCTACACGGCCTCCCTCCCCGCCAAGCTGAGGCAGTACTCGGACTTCCTC

 141  G  S  R  P  W  F  A  G  D  K  L  T  Y  I  D  F  L  A  Y  E
 421  GGCAGCAGACCCTGGTTCGCCGGAGACAAGCTAACCTACATCGACTTCCTGGCCTACGAG

 161  I  F  D  Q  H  L  S  L  D  R  T  C  L  D  G  F  K  N  L  Q
 481  ATCTTCGACCAGCACCTCAGCCTCGACCGCACTTGCCTCGACGGCTTCAAGAACCTCCAG

 181  A  F  Q  K  R  F  E  D  L  E  A  I  K  K  Y  M  A  S  P  K
 541  GCCTTCCAGAAGAGGTTTGAGGATCTCGAGGCCATCAAGAAGTACATGGCGTCGCCGAAG

 201  F  L  K  K  P  I  C  N  K  Y  A  Q  F  T  I  I  E  G  K  -
 601  TTCCTCAAAAAACCGATTTGCAACAAGTACGCTCAGTTTACGATCATTGAGGGAAAATGA
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Corrección a la secuencia de cDNA de la GST clase mu de *Litopenaues vannamei* publicada en el artículo: A mu-class Glutathione S-Transferase from the Marine Shrimp *Litopenaeus vannamei*: Molecular Cloning and Active-Site Structural Modeling, Carmen A. Contreras-Vergara, Citlalli Harris-Valle, Rogerio R. Sotelo-Mundo, and Gloria Yepiz-Plascencia. Journal of Biochemical and Molecular Toxicology, Vol. 18(5), 2004.

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   1  M  L  P  V  L  G  Y  W  K  T  R  A  L  C  Q  P  I  R  L  M
   1  ATGCTGCCCGTTTTAGGCTACTGGAAAACTCGGGCGCTCTGCCAGCCCATTCGGCTGATG

  21  L  G  Y  T  G  T  E  F  E  E  K  N  Y  P  V  G  D  A  P  D
  61  CTGGGGTACACGGGCACTGAGTTCGAGGAGAAAAACTACCCTGTCGGCGACGCTCCAGAC

  41  Y  D  K  S  E  W  L  A  V  K  F  K  L  G  L  A  F  P  N  L
 121  TACGACAAGAGCGAATGGCTGGCCGTCAAGTTCAAACTCGGCCTCGCCTTCCCCAACCTT

  61  P  Y  Y  I  D  G  D  V  K  I  T  Q  S  K  A  I  M  R  Y  L
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  81  A  R  K  H  G  L  C  G  T  T  P  E  E  L  V  R  T  D  M  I
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 101  E  C  Q  L  T  D  M  H  E  A  F  F  T  V  T  Y  E  H  Y  E
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 201  F  L  K  K  P  I  C  N  K  Y  A  Q  F  T  I  I  E  G  K  -
 601  TTCCTCAAAAAACCGATTTGCAACAAGTACGCTCAGTTTACGATCATTGAGGGAAAATGA
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